MORPHOMETRIC DATA SHEET

		REMARKS
Brand No.		
Date		
Sex		
Weight (Kg.)		
Total Length (CM.)		
Snout - Eye		
Snout - Ear		
Snout - Anterior Origin of Flipper		
Snout - Origin of Melon		
Snout - Angle of Jaw		
Snout - Blowhole		
Snout - Origin of Dorsal Fin		
Flipper - (Origin - Tip)		
Flipper - (Width)		
Fluke (Width)		
Height of Dorsal Fin		
Girth (Origin of Dorsal)		
Girth (Umbilicus)		

Comments:

FIGURE 4: FORM USED TO LOG MORPHOMETRIC DATA FOR EACH CAPTURED INDIVIDUAL.

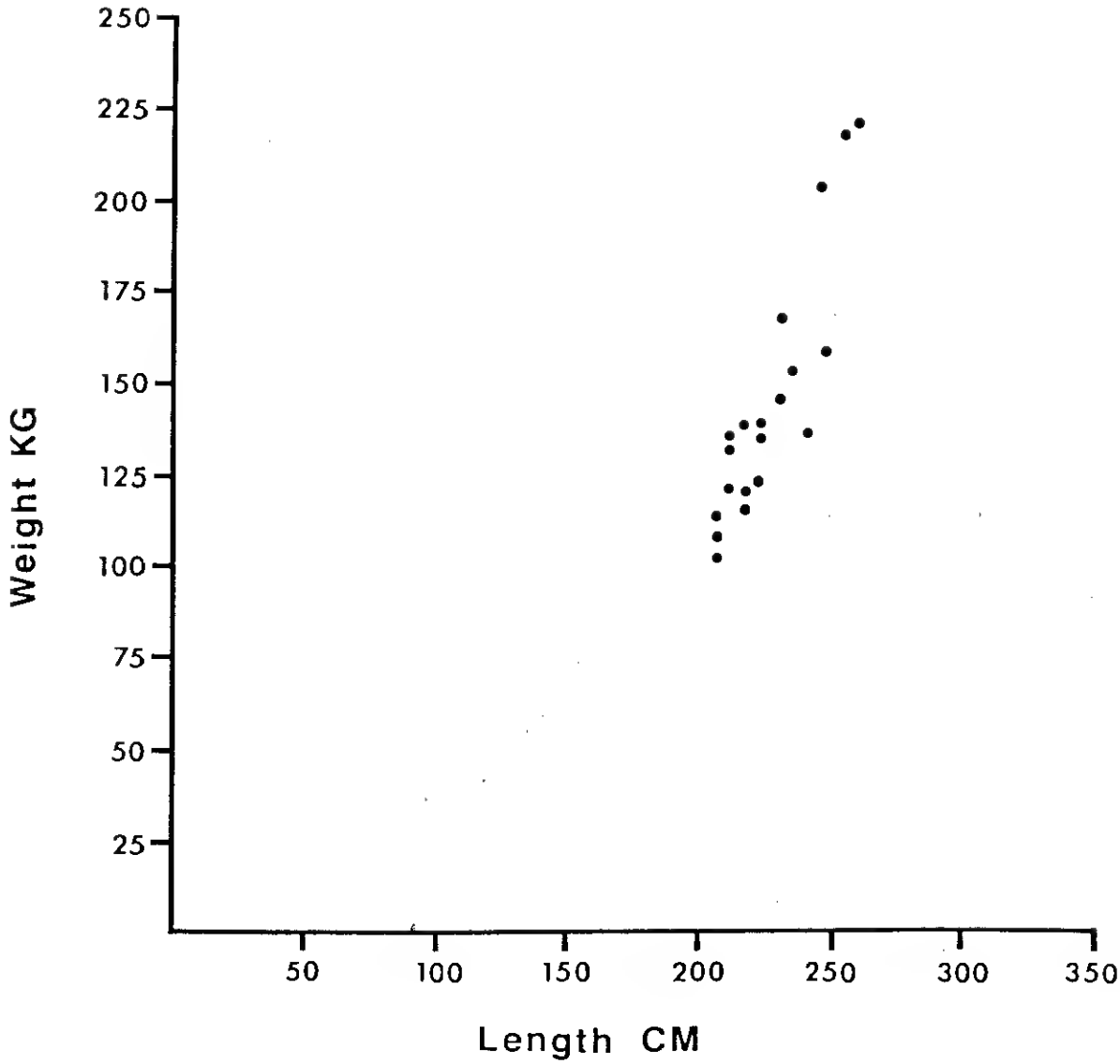

FIGURE 5: LENGTH-WEIGHT RELATIONSHIP OF MALE ATLANTIC BOTTLENOSE DOLPHINS SAMPLED FROM THE MISSISSIPPI SOUND IN 1982

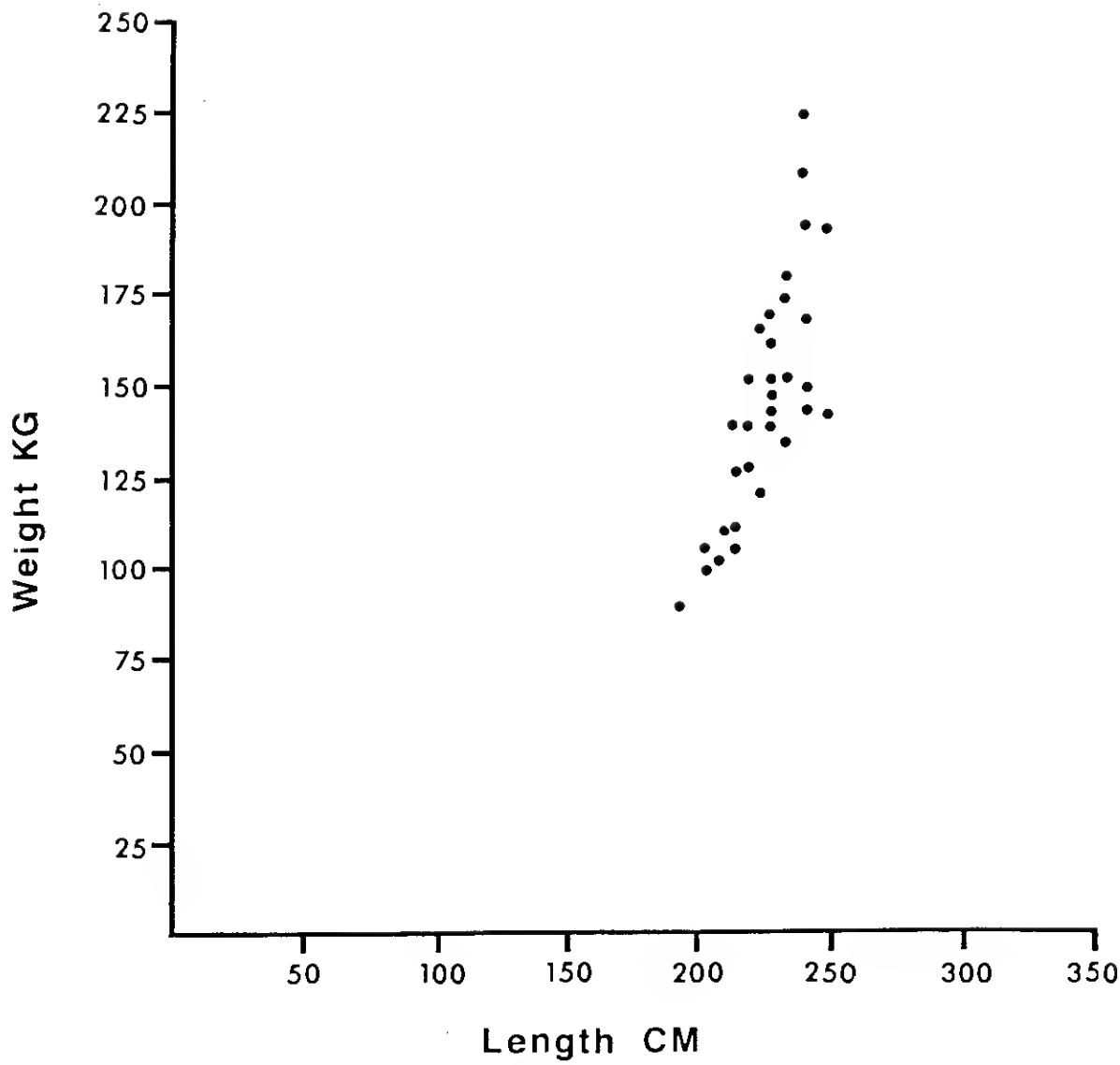

FIGURE 6: LENGTH-WEIGHT RELATIONSHIP OF FEMALE Tursiops truncatus COLLECTED FROM THE MISSISSIPPI SOUND IN 1982.

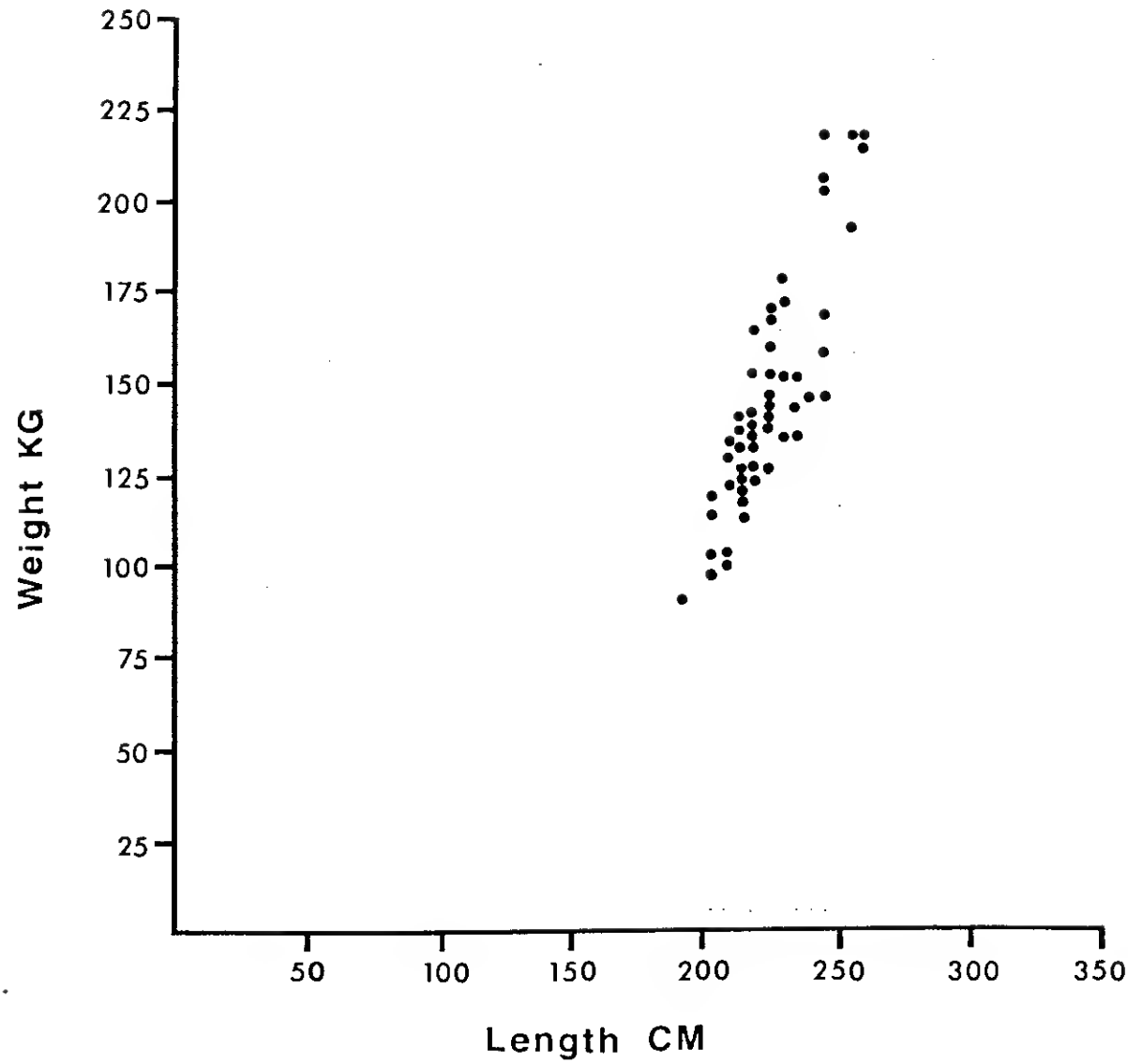

FIGURE 7: SCATTERGRAM OF LENGTHS AND WEIGHTS OF ALL (MALE AND FEMALE) DOLPHINS SAMPLED IN 1982.

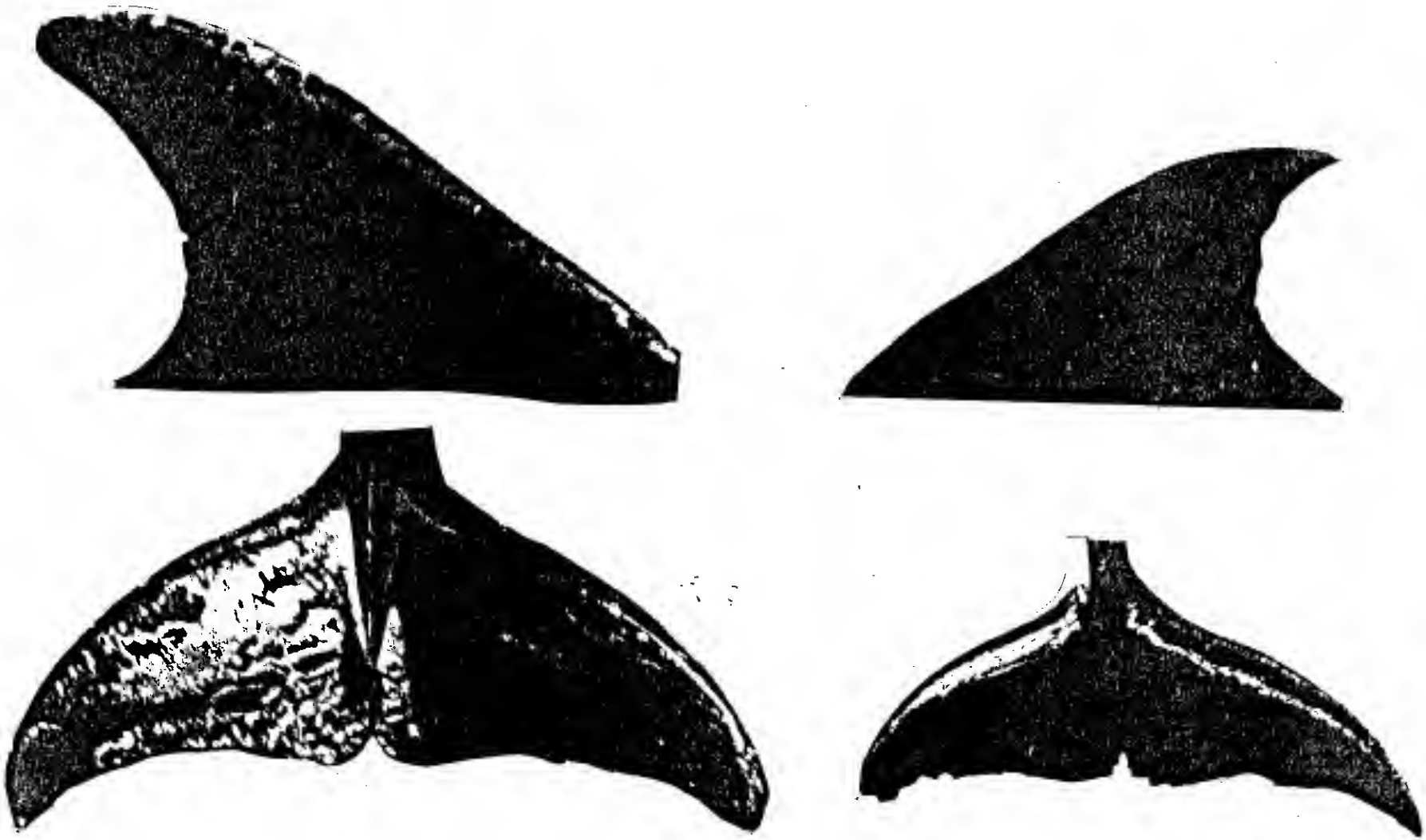

Figure 8: Photographic profile of dorsal fin and fluke of animal no. 601 on the left and animal no. 602 on the right.

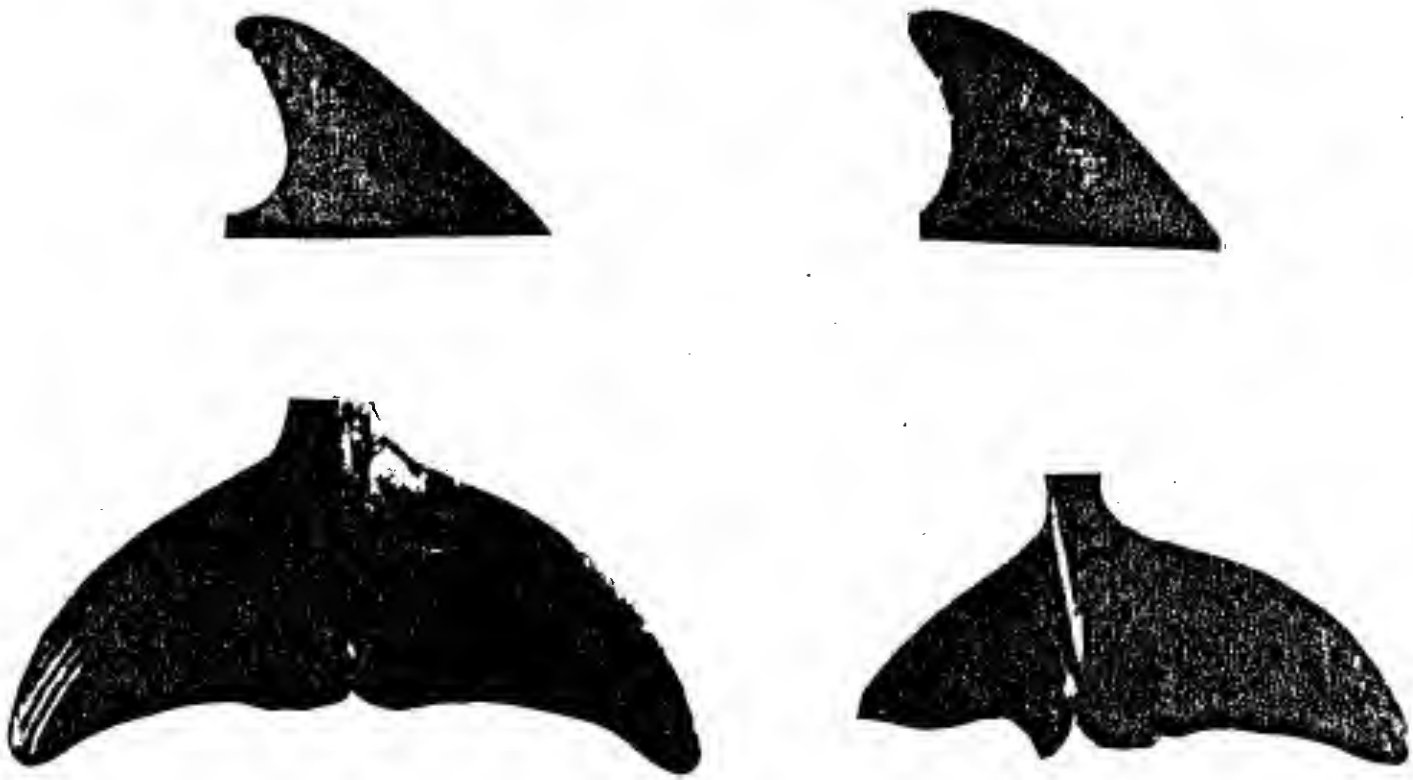

Figure 9: Photographic profile of dorsal fin and fluke of animal no. 603 on the left and animal no. 604 on the right.

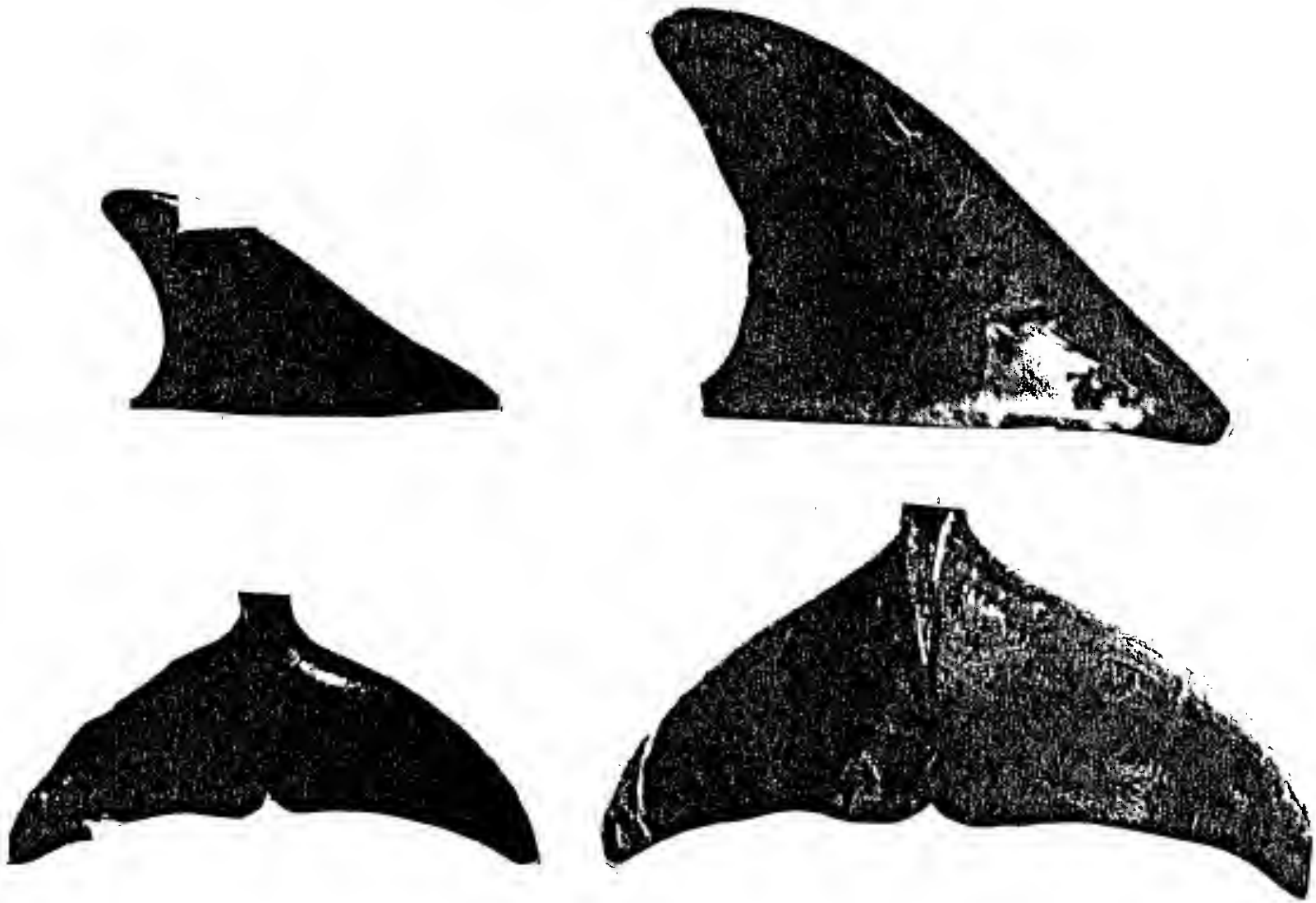

Figure 10. Photographic profile of dorsal fin and fluke of animal no. 605 on the left and animal no. 606 on the right.

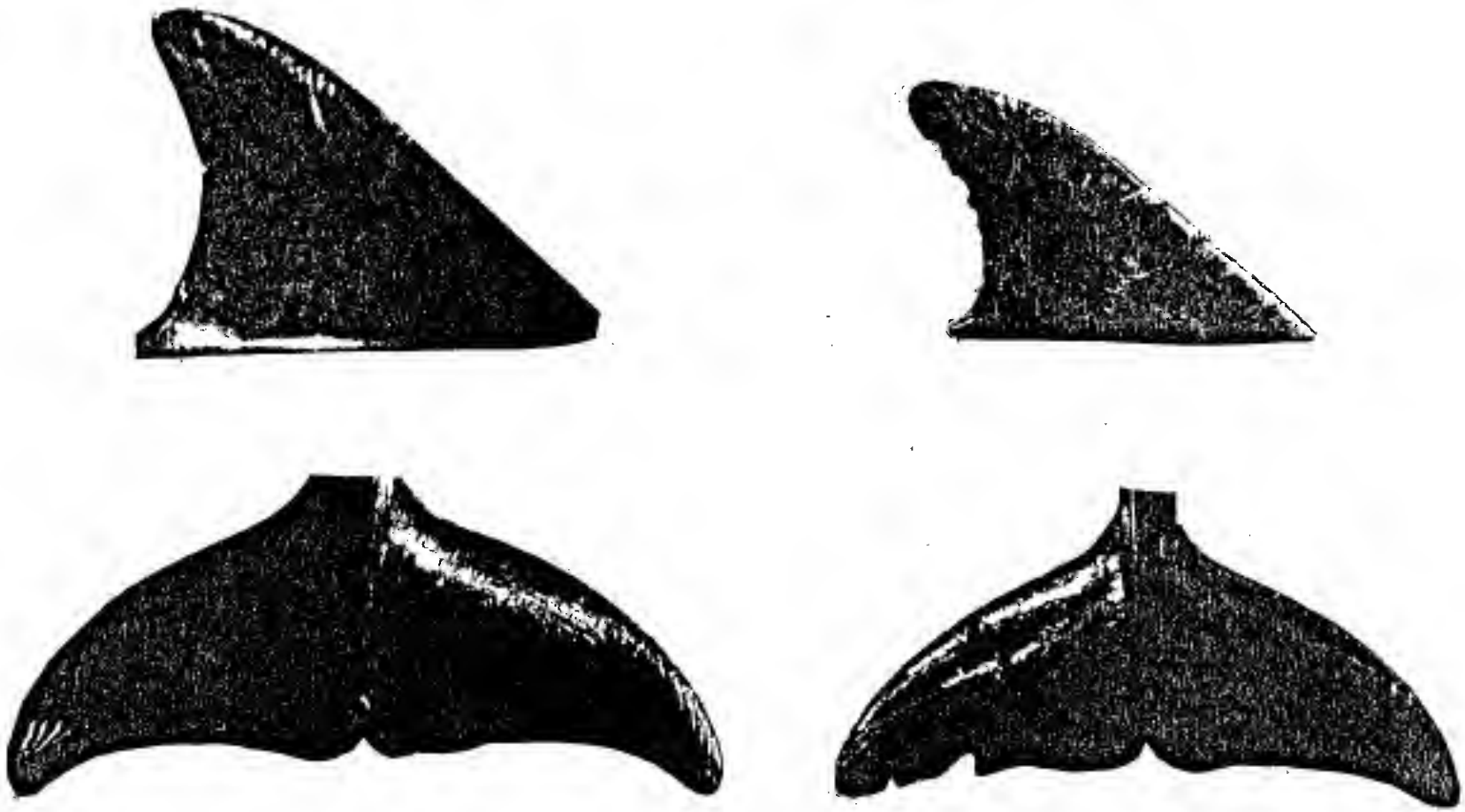

Figure 11. Photographic profile of dorsal fin and fluke of animal no. 607 on the left and animal no. 608 on the right.

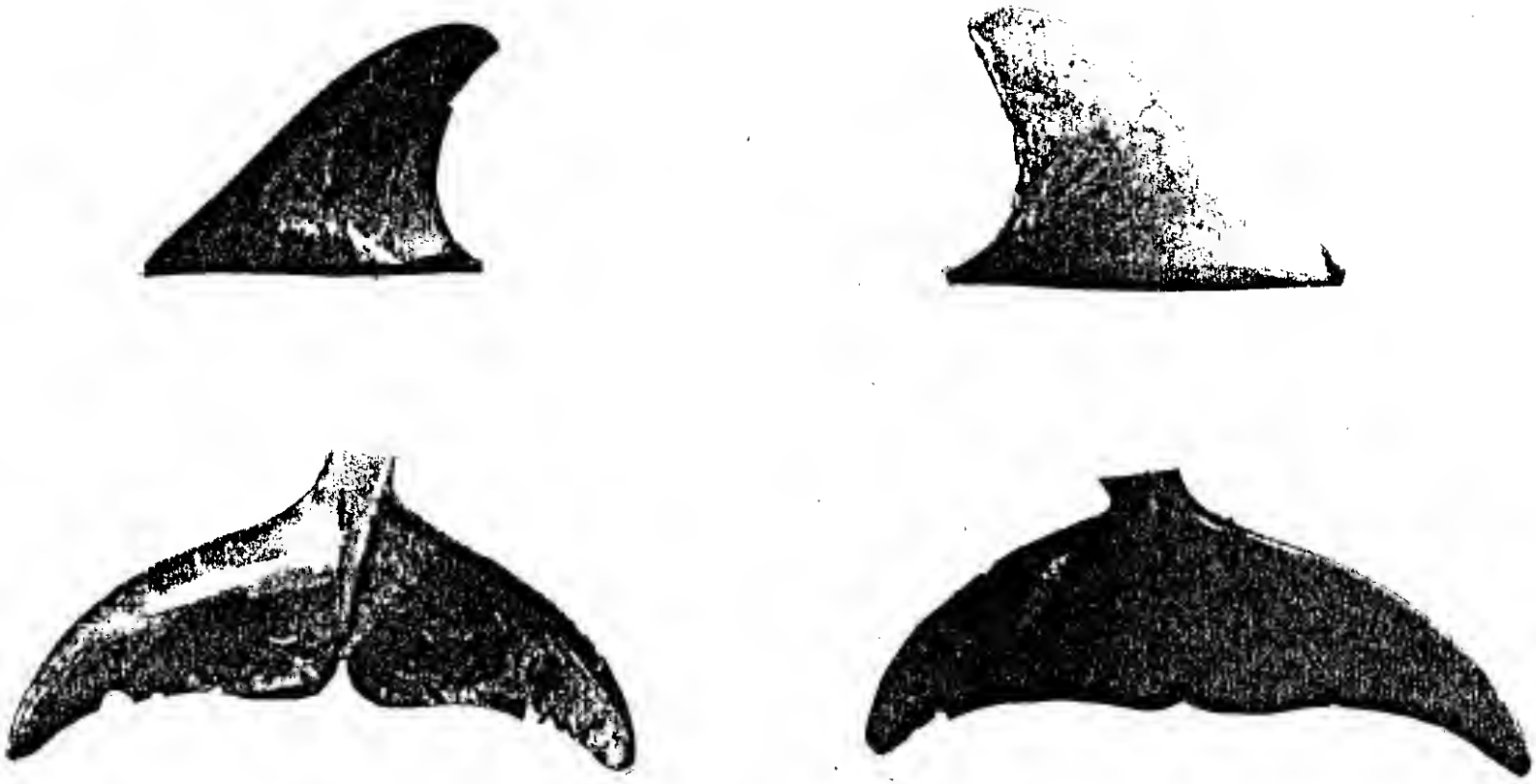

Figure 12. Photographic profile of dorsal fin and fluke of animal no. 609 on the left and animal no. 610 on the right.

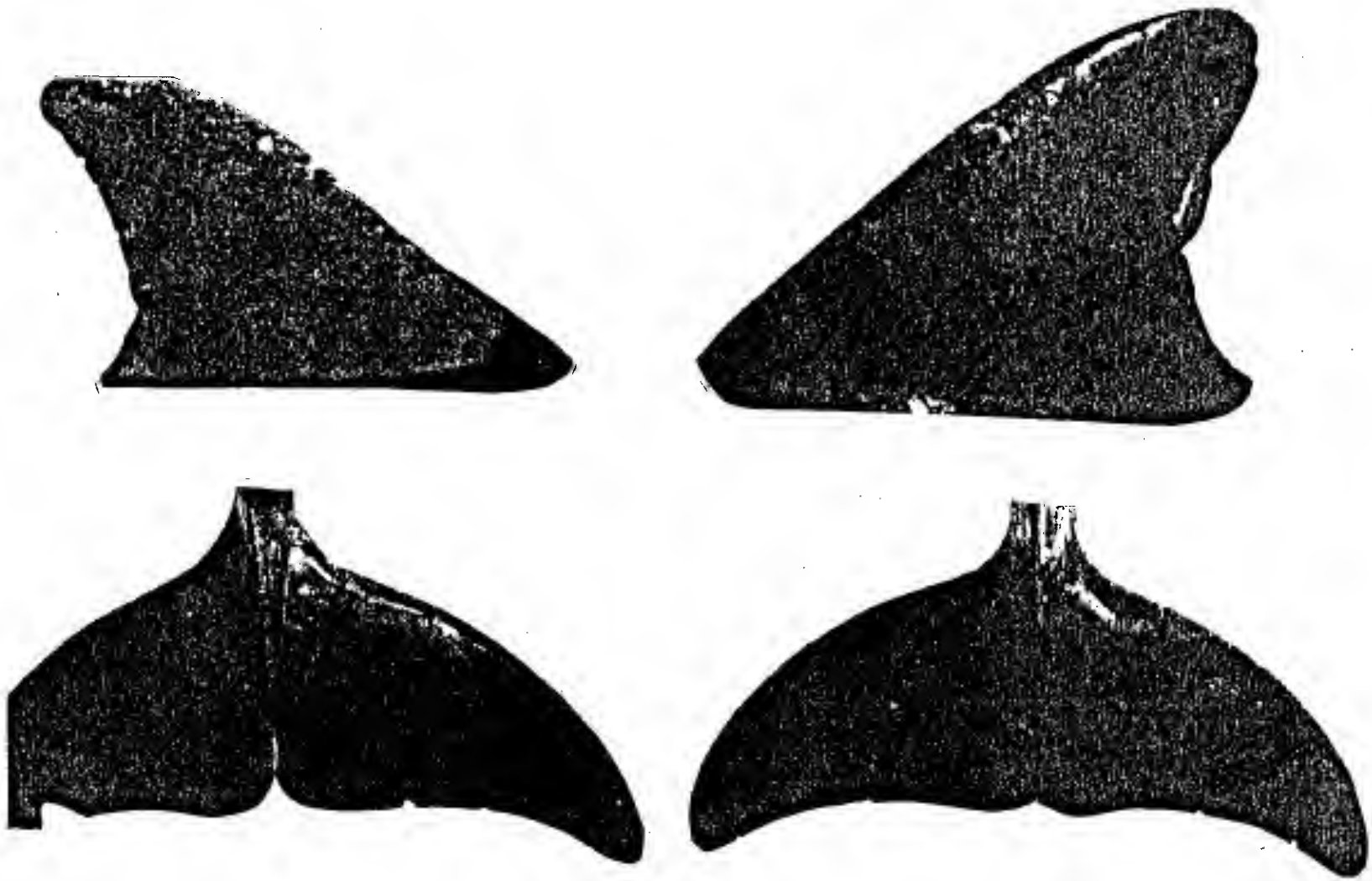

Figure 13. Photographic profile of dorsal fin and fluke of animal no. 611 on the left and animal no. 612 on the right.

NOTE: Cuts on the fin and fluke of animal no. 611 are artifacts.

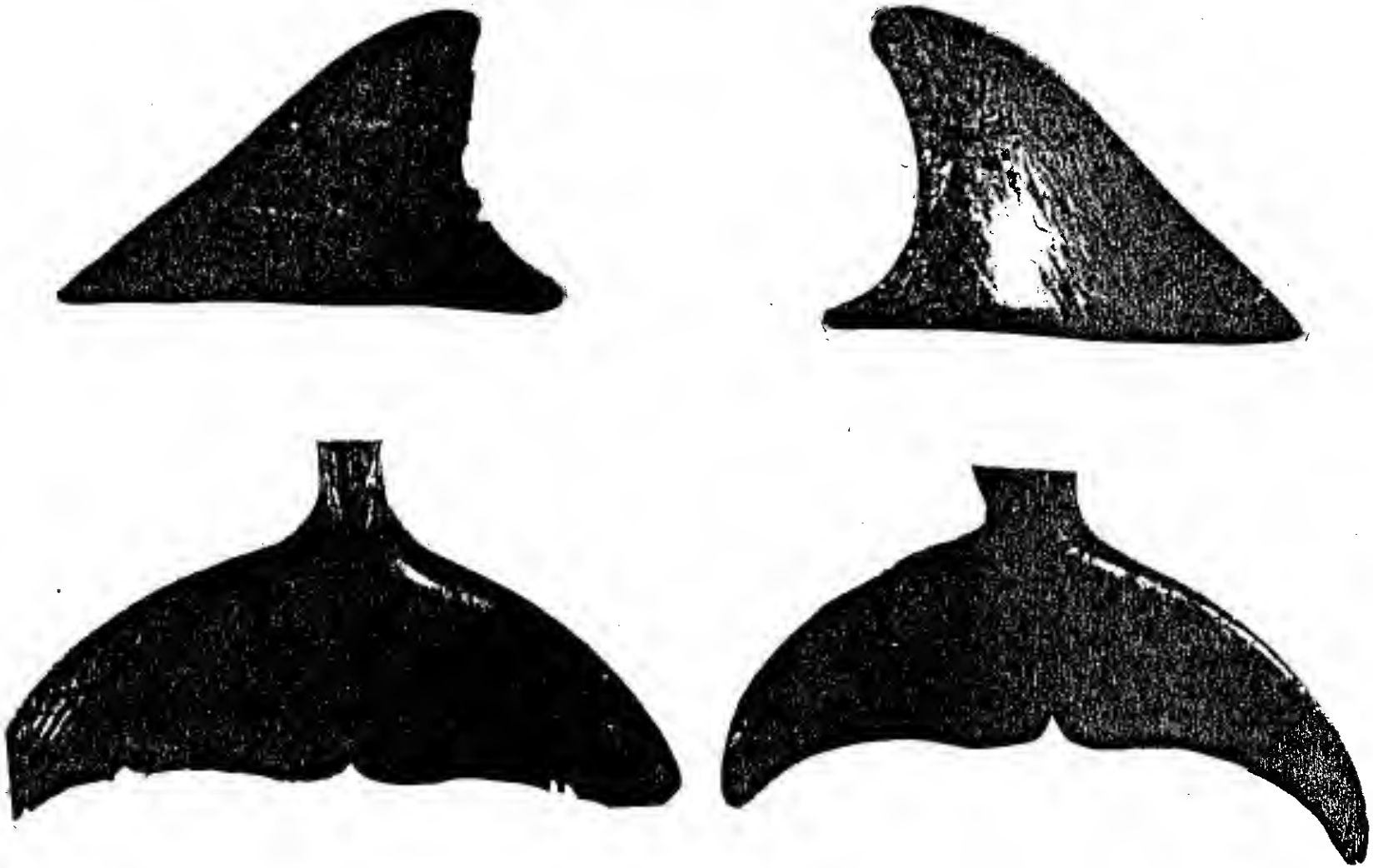

Figure 14. Photographic profile of dorsal fin and fluke of animal no. 613 on the left and animal no. 614 on the right.

NOTE: Cut on fluke of animal no. 613 is an artifact.

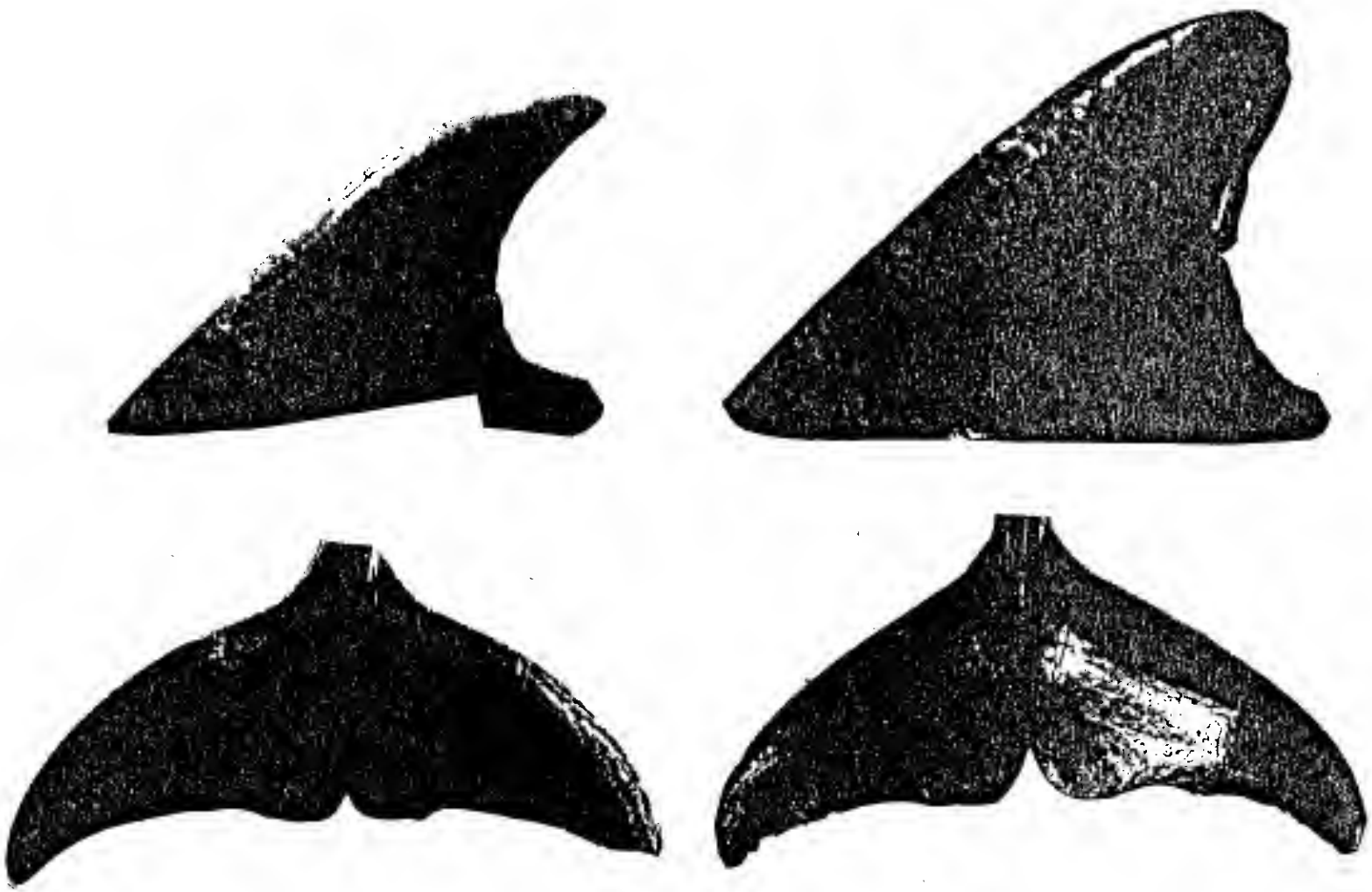

Figure 15. Photographic profile of dorsal fin and fluke of animal no. 615 on the left and animal no. 618 on the right.

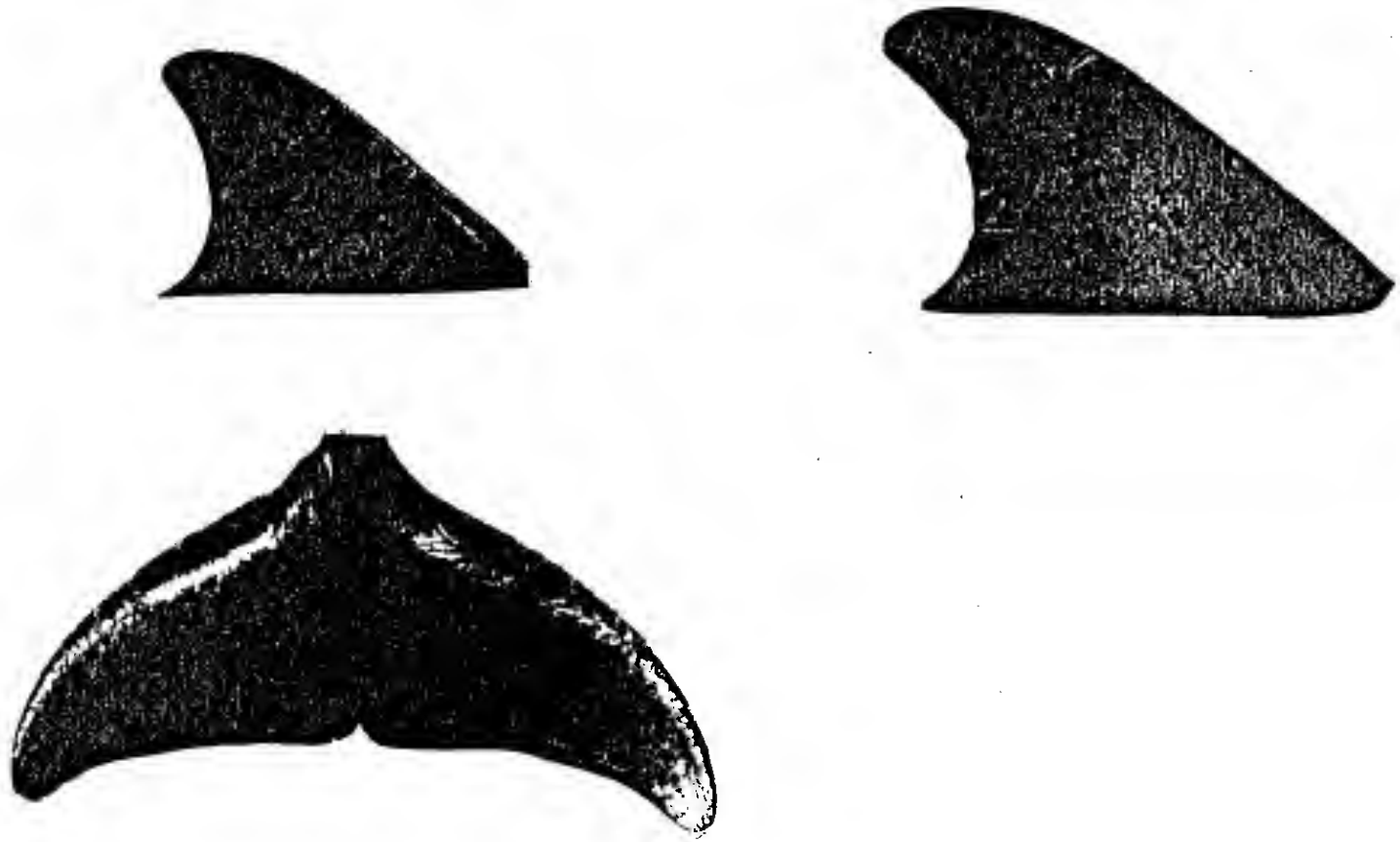

Figure 16. Photographic profile of dorsal fin and fluke of animal no. 619 on the left and only the dorsal fin of animal no. 620 on the right.

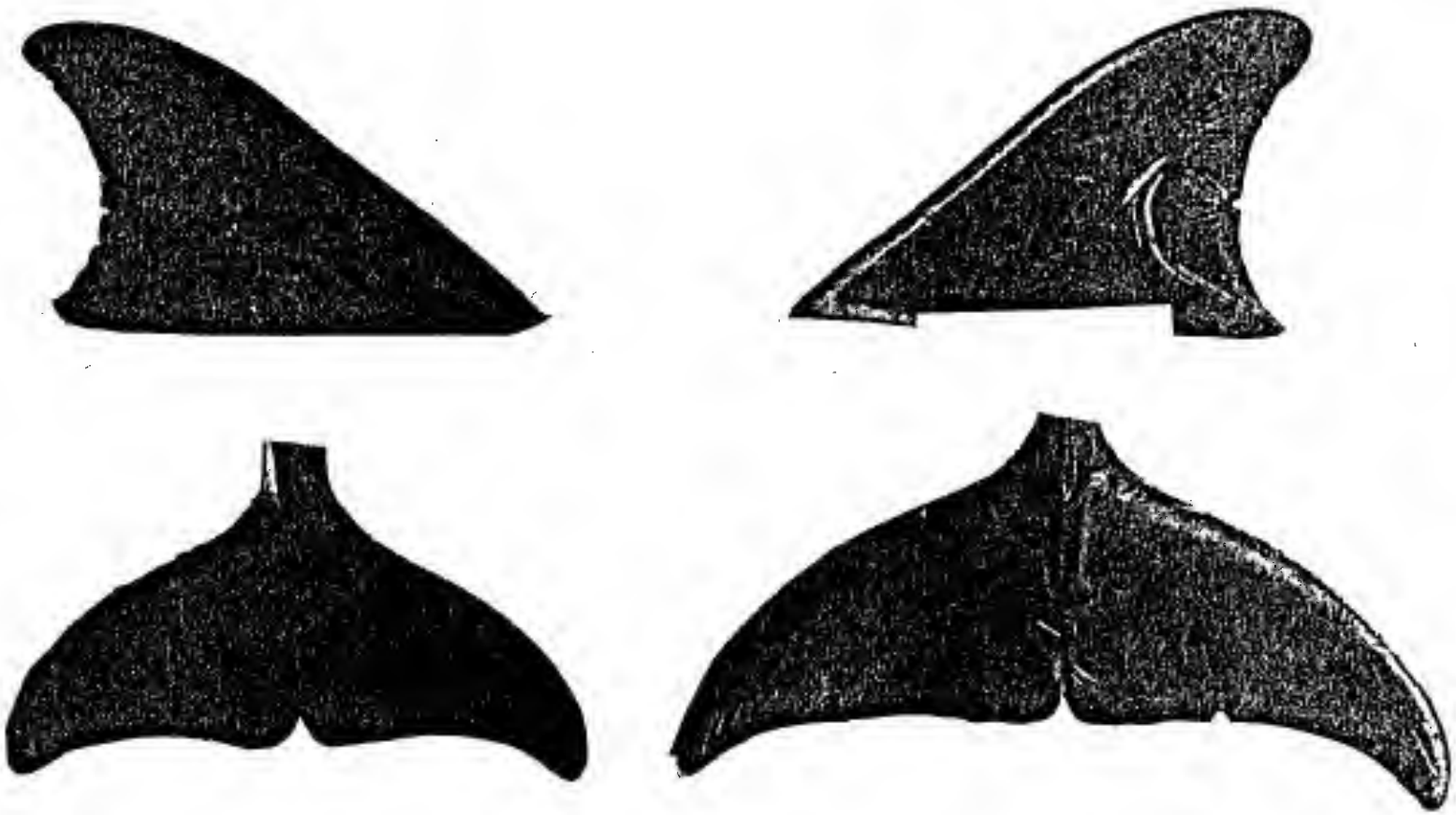

Figure 17. Photographic profile of dorsal fin and fluke of animal no. 621 on the left and animal no. 622 on the right.

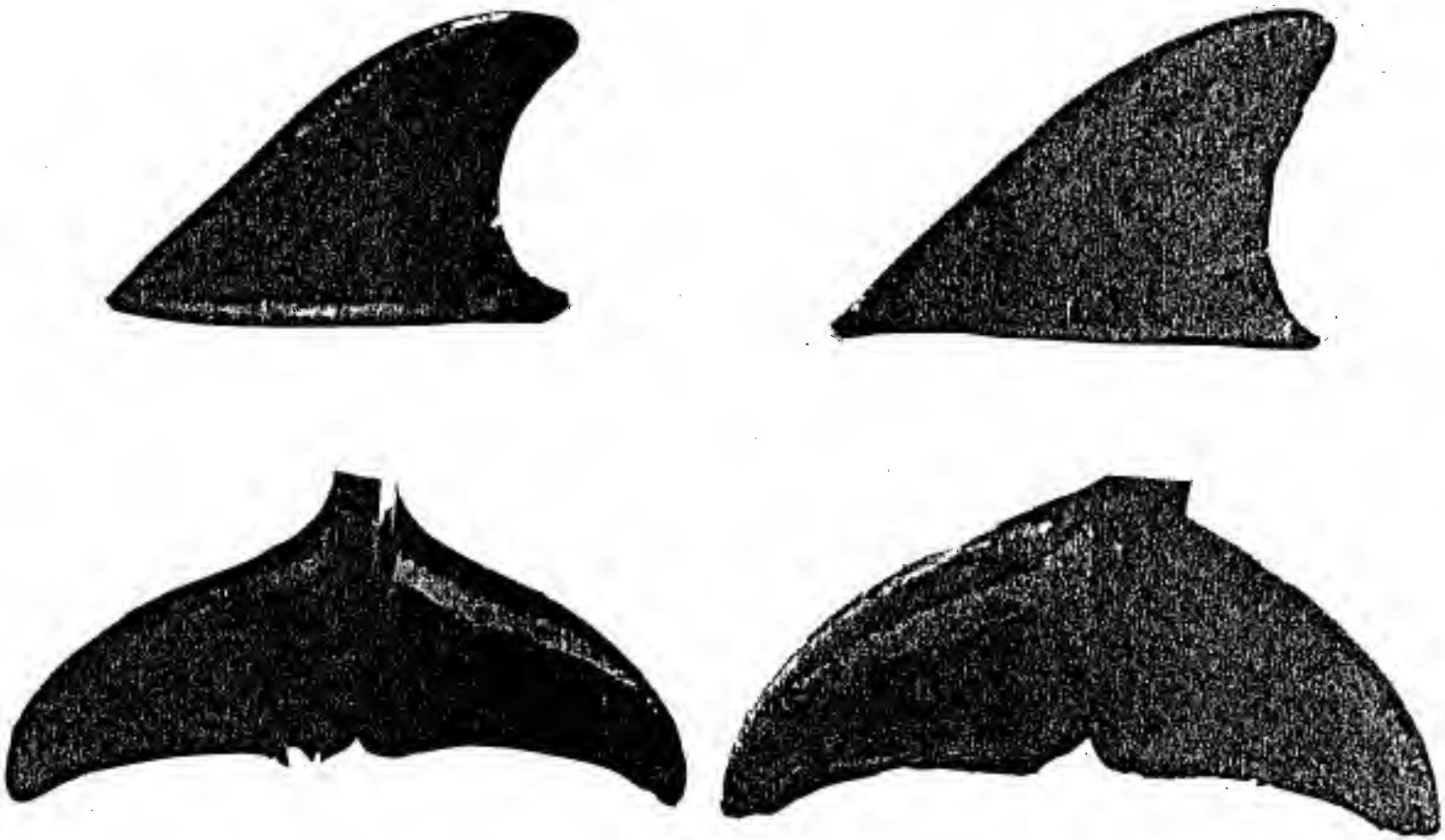

Figure 18. Photographic profile of dorsal fin and fluke of animal no. 623 on the left and animal no. 624 on the right.

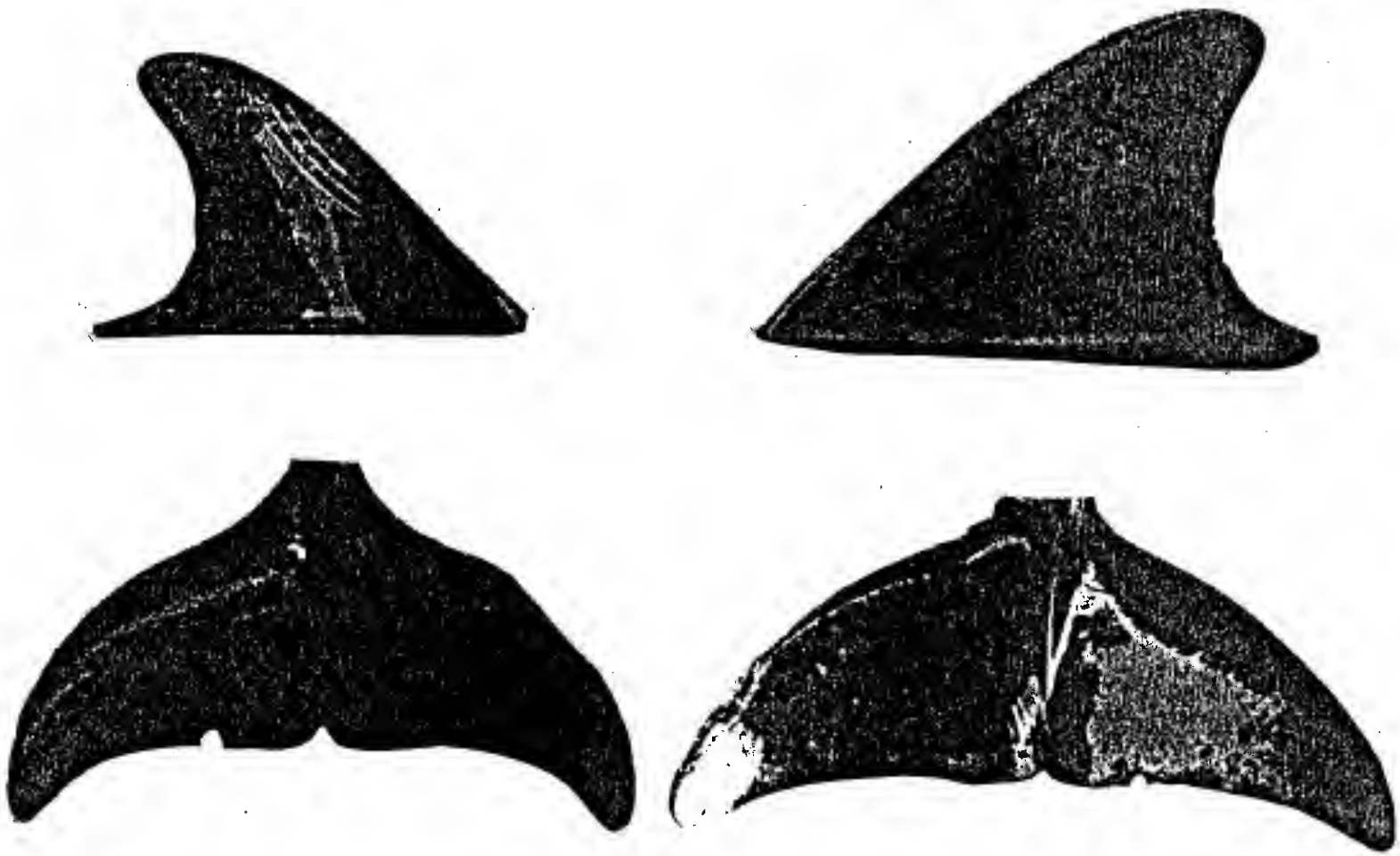

Figure 19. Photographic profile of dorsal fin and fluke of animal no. 626 on the left and animal no. 627 on the right.

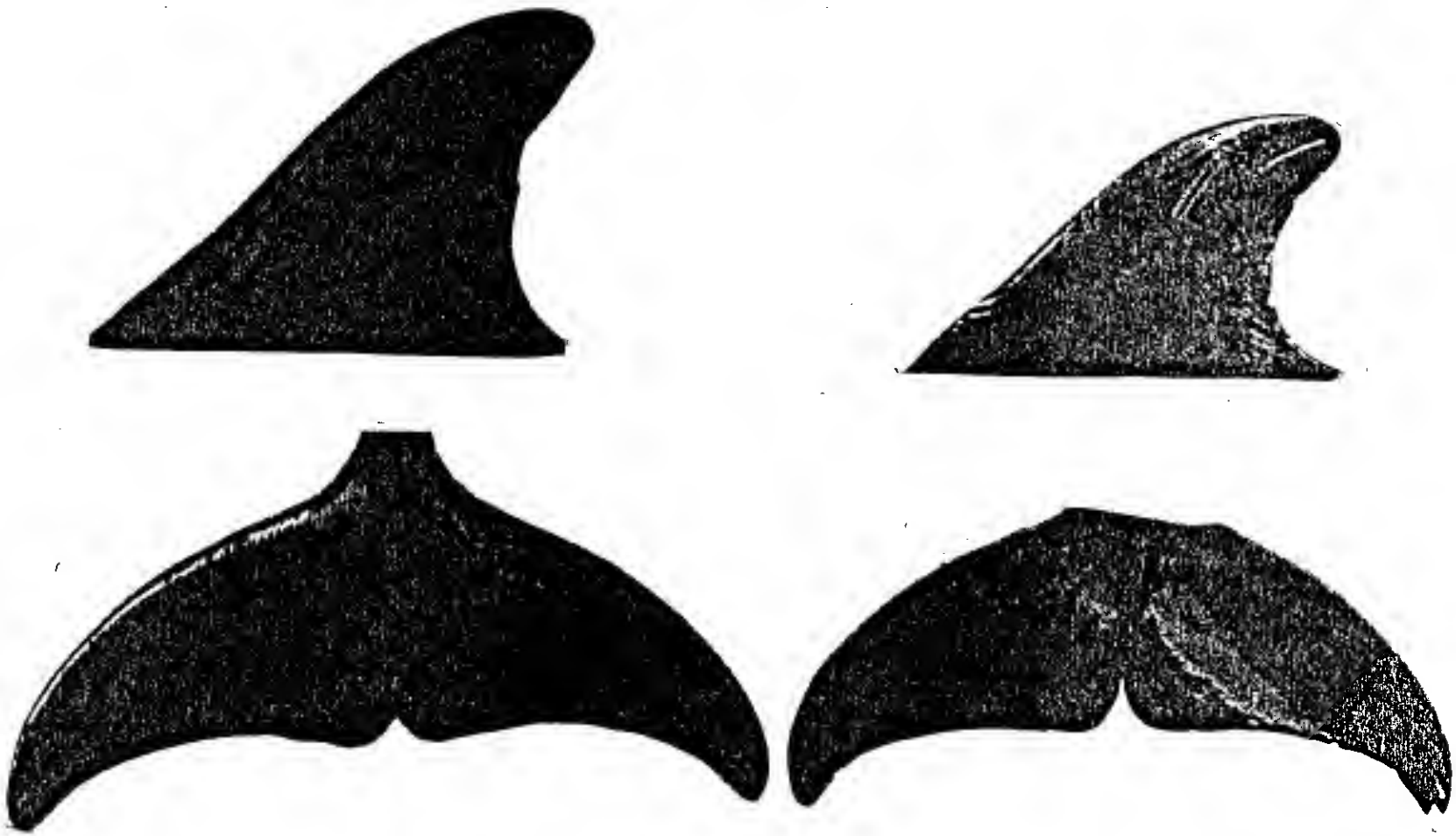

Figure 20. Photographic profile of dorsal fin and fluke of animal no. 628 on the left and animal no. 629 on the right.

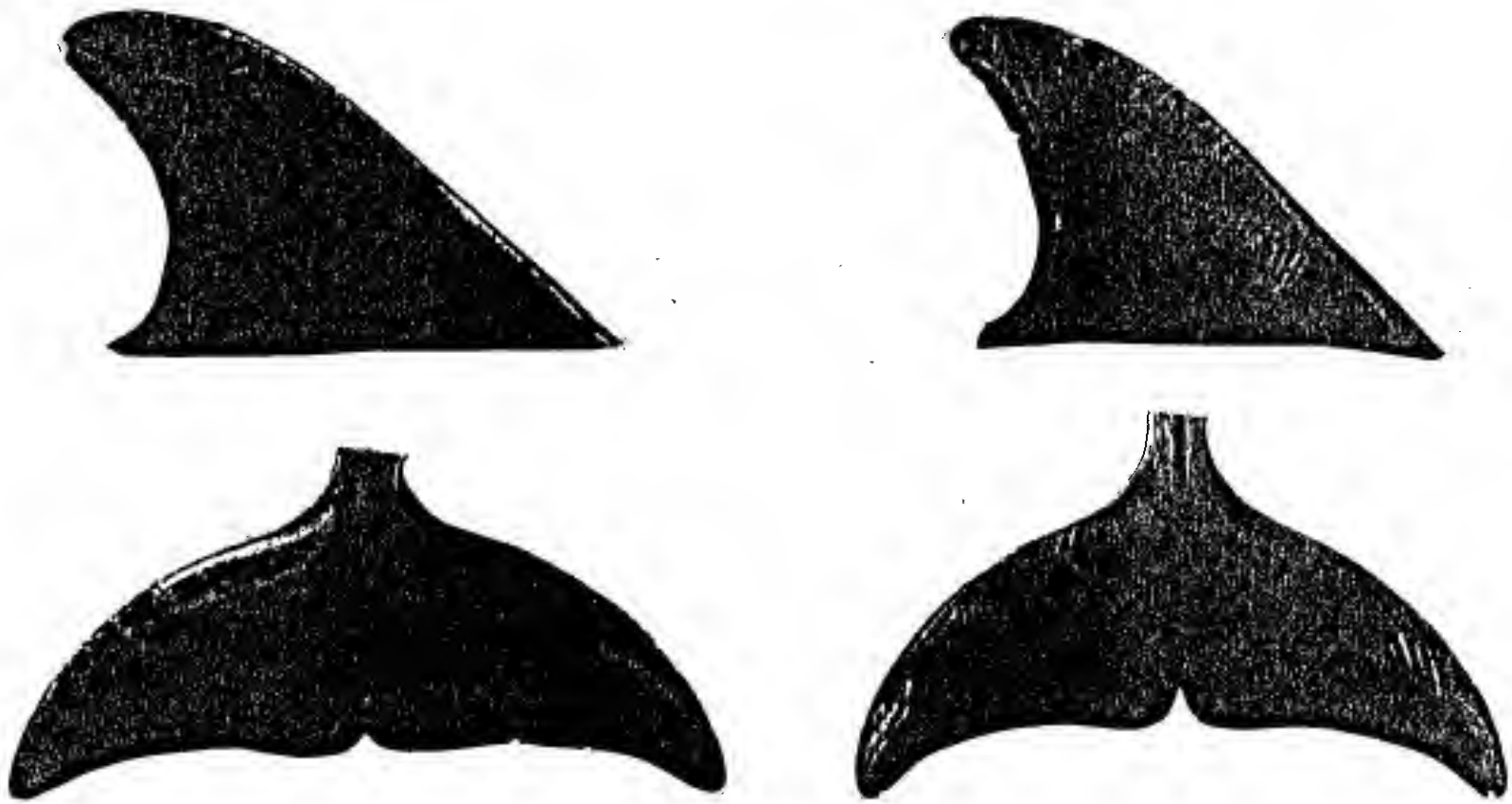

Figure 21. Photographic profile of dorsal fin and fluke of animal no. 630 on the left and animal no. 631 on the right.

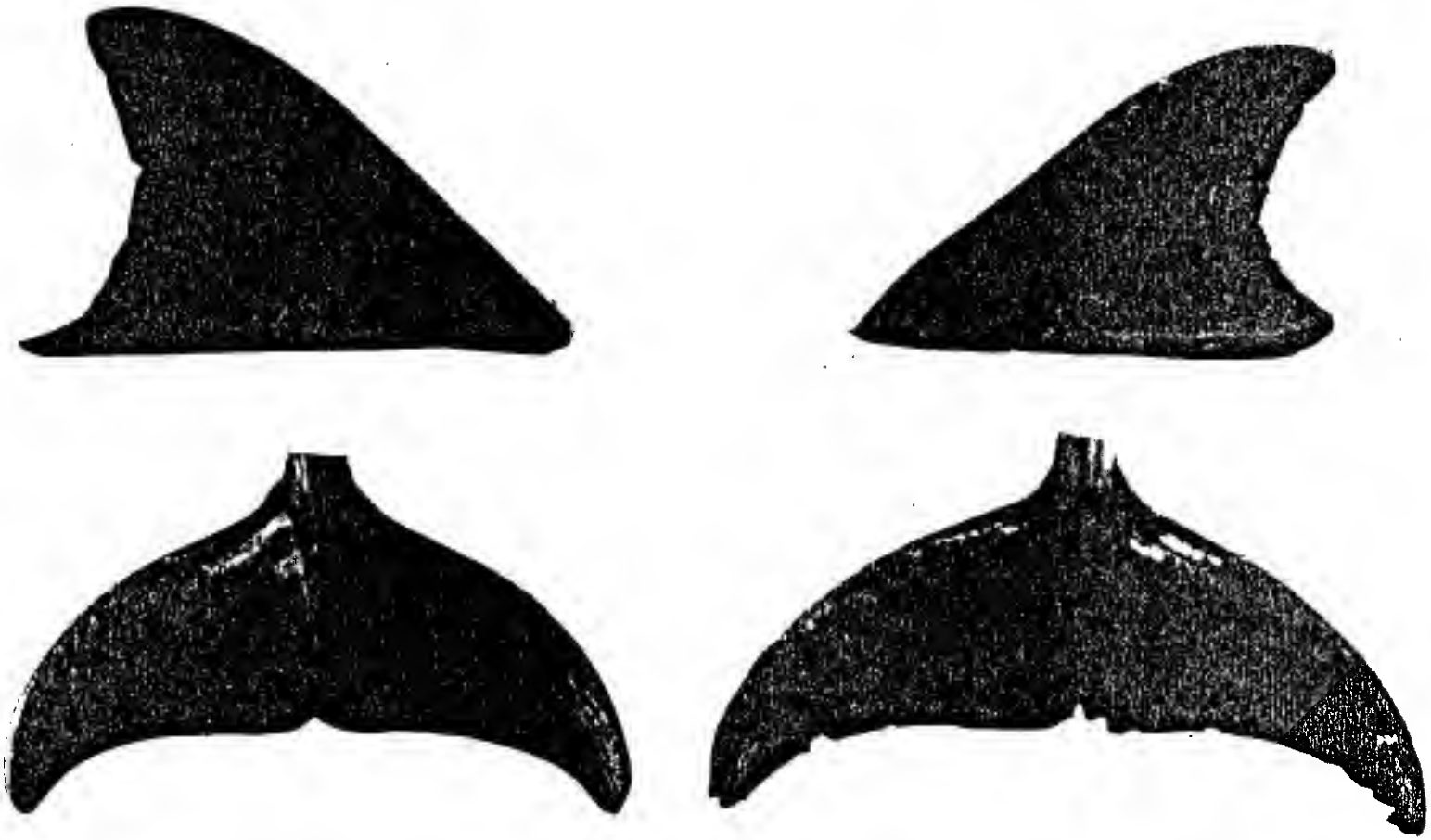

Figure 22. Photographic profile of dorsal fin and fluke of animal no. 632 on the left and animal no. 633 on the right.

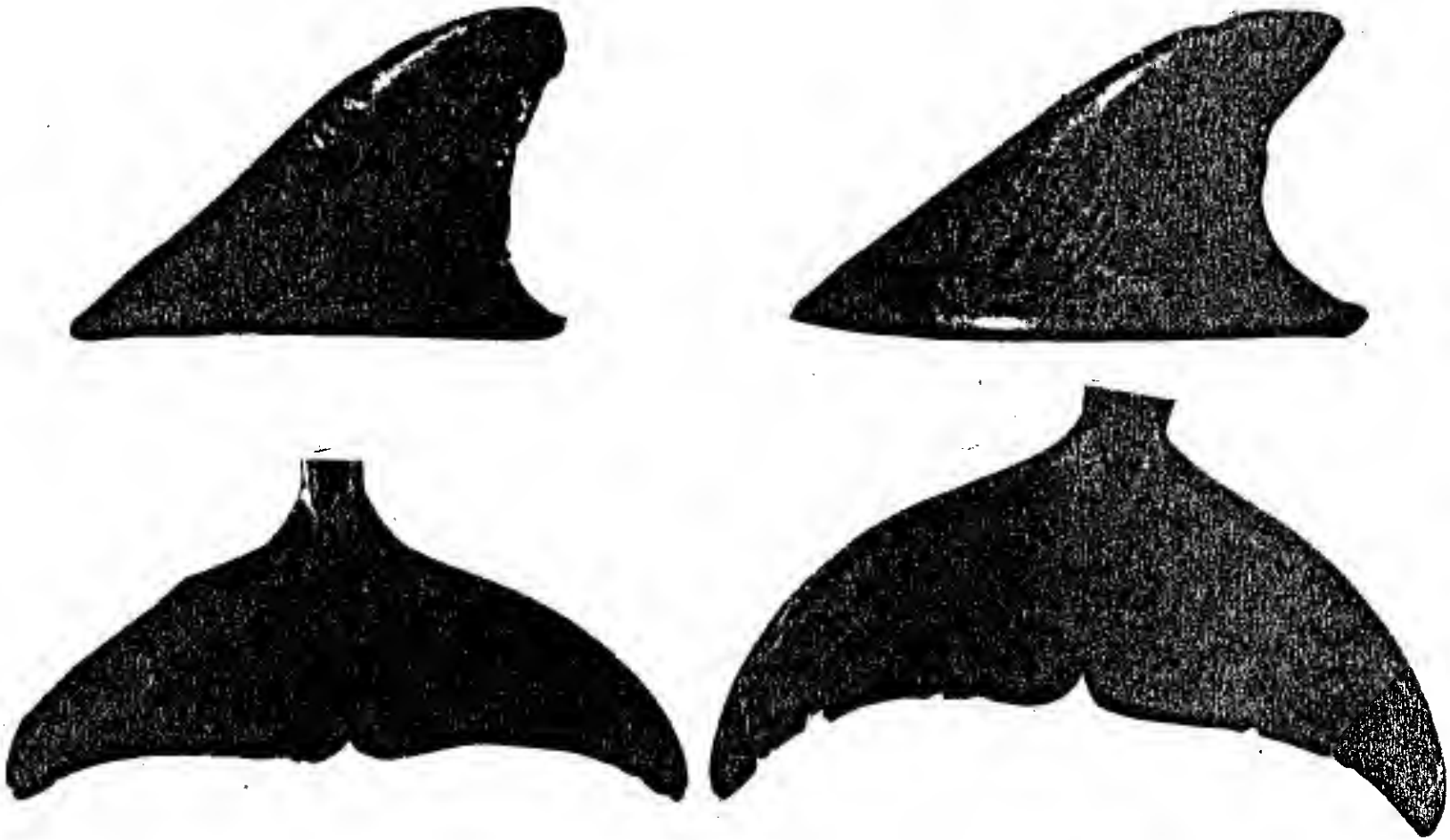

Figure 23. Photographic profile of dorsal fin and fluke of animal no. 634 on the left and animal no. 635 on the right.

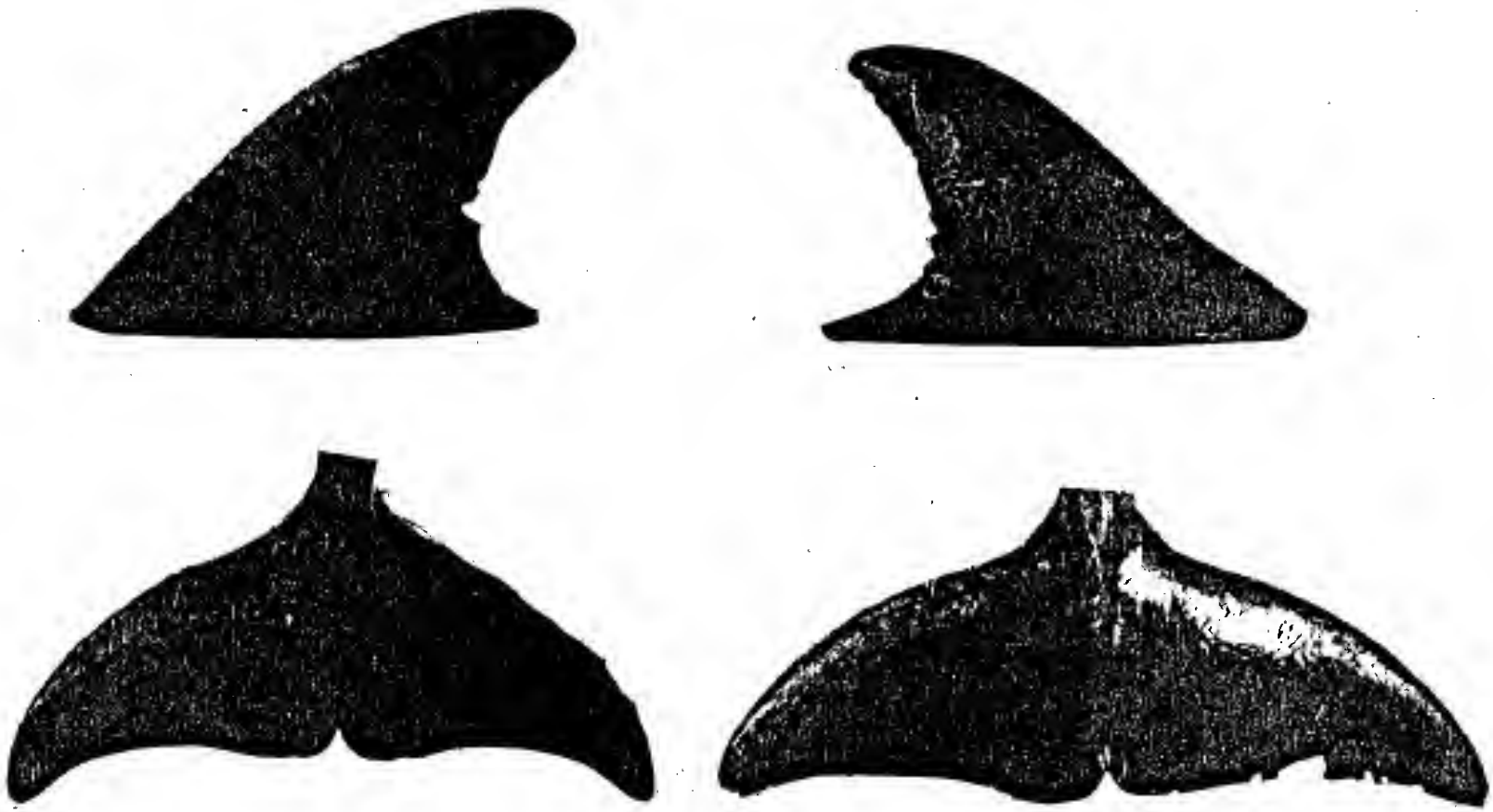

Figure 24. Photographic profile of dorsal fin and fluke of animal no. 636 on the left and animal no. 637 on the right.

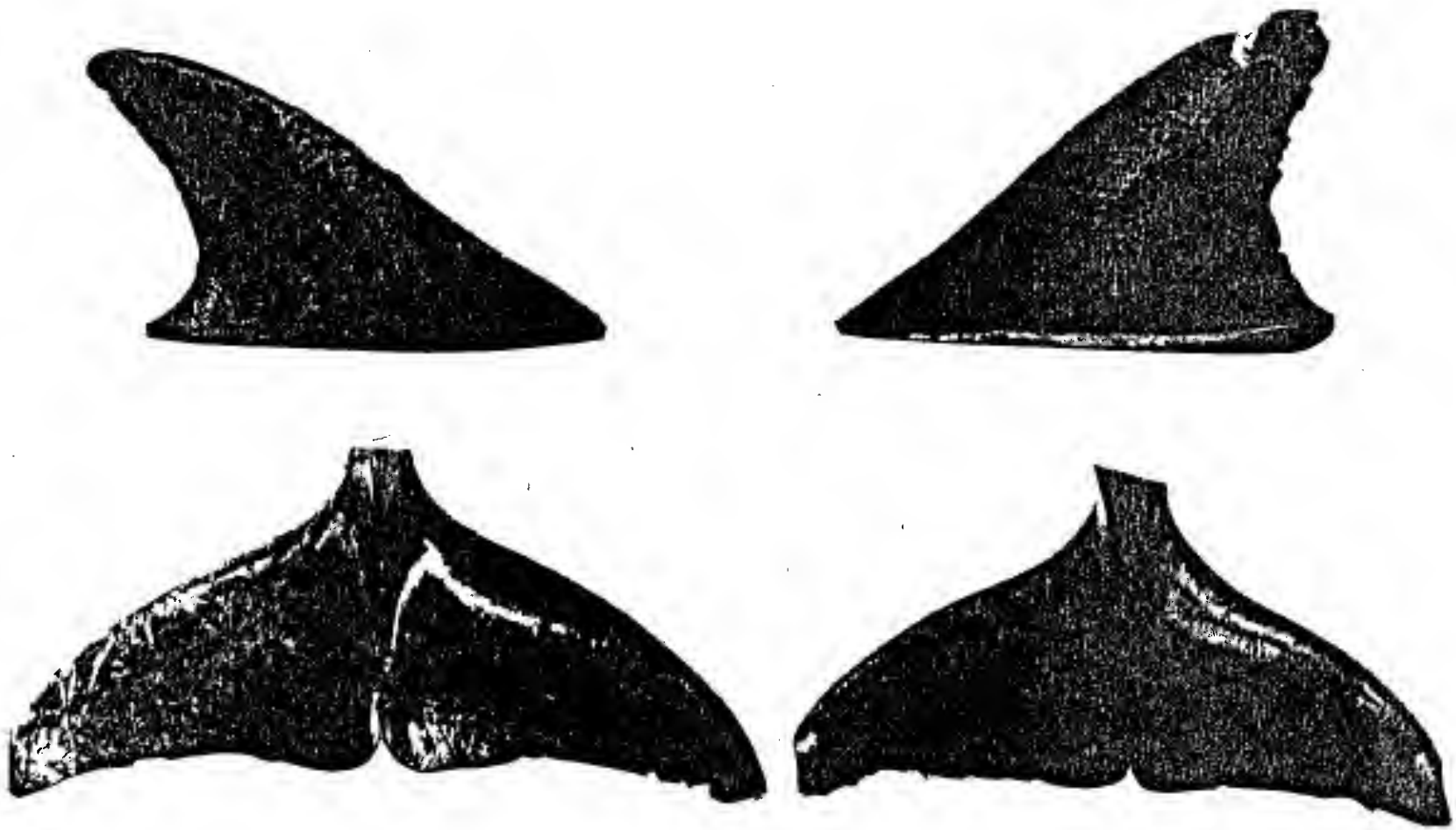

Figure 25. Photographic profile of dorsal fin and fluke of animal no. 638 on the left and animal no. 639 on the right.

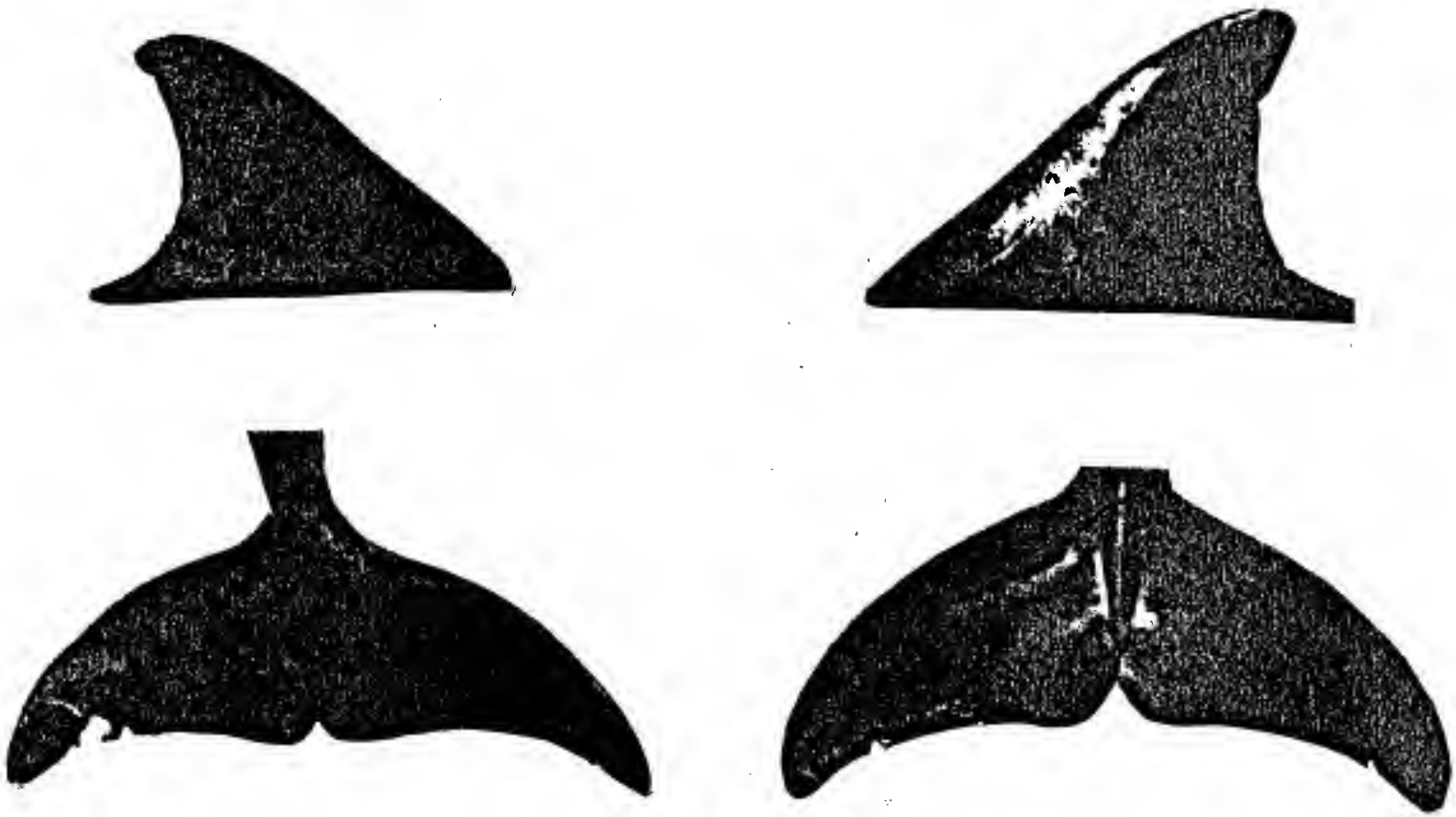

Figure 26. Photographic profile of dorsal fin and fluke of animal no. 640 on the left and animal no. 641 on the right.

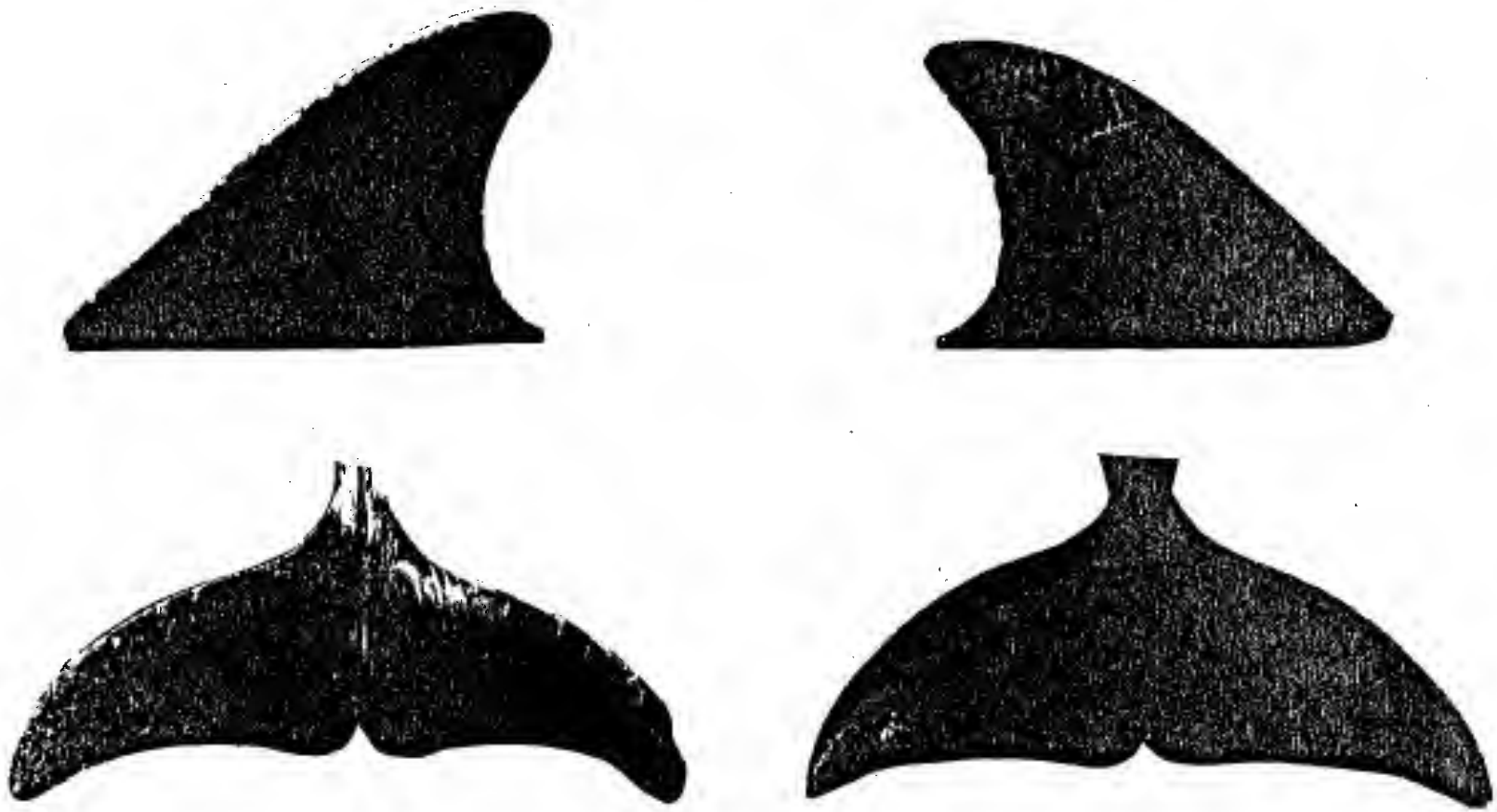

Figure 27. Photographic profile of dorsal fin and fluke of animal no. 642 on the left and animal no. 643 on the right.

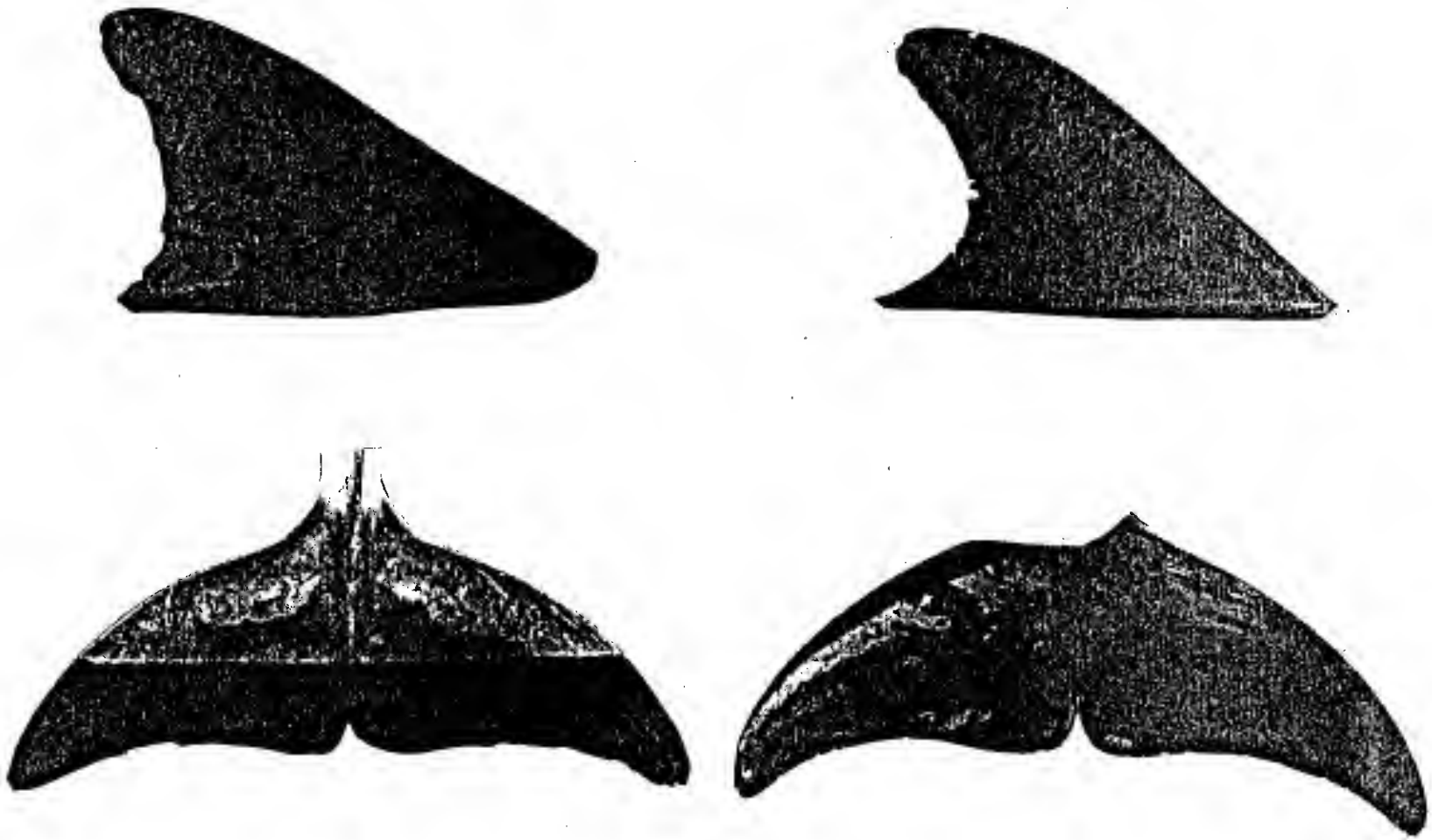

Figure 28. Photographic profile of dorsal fin and fluke of animal no. 644 on the left and animal no. 645 on the right.

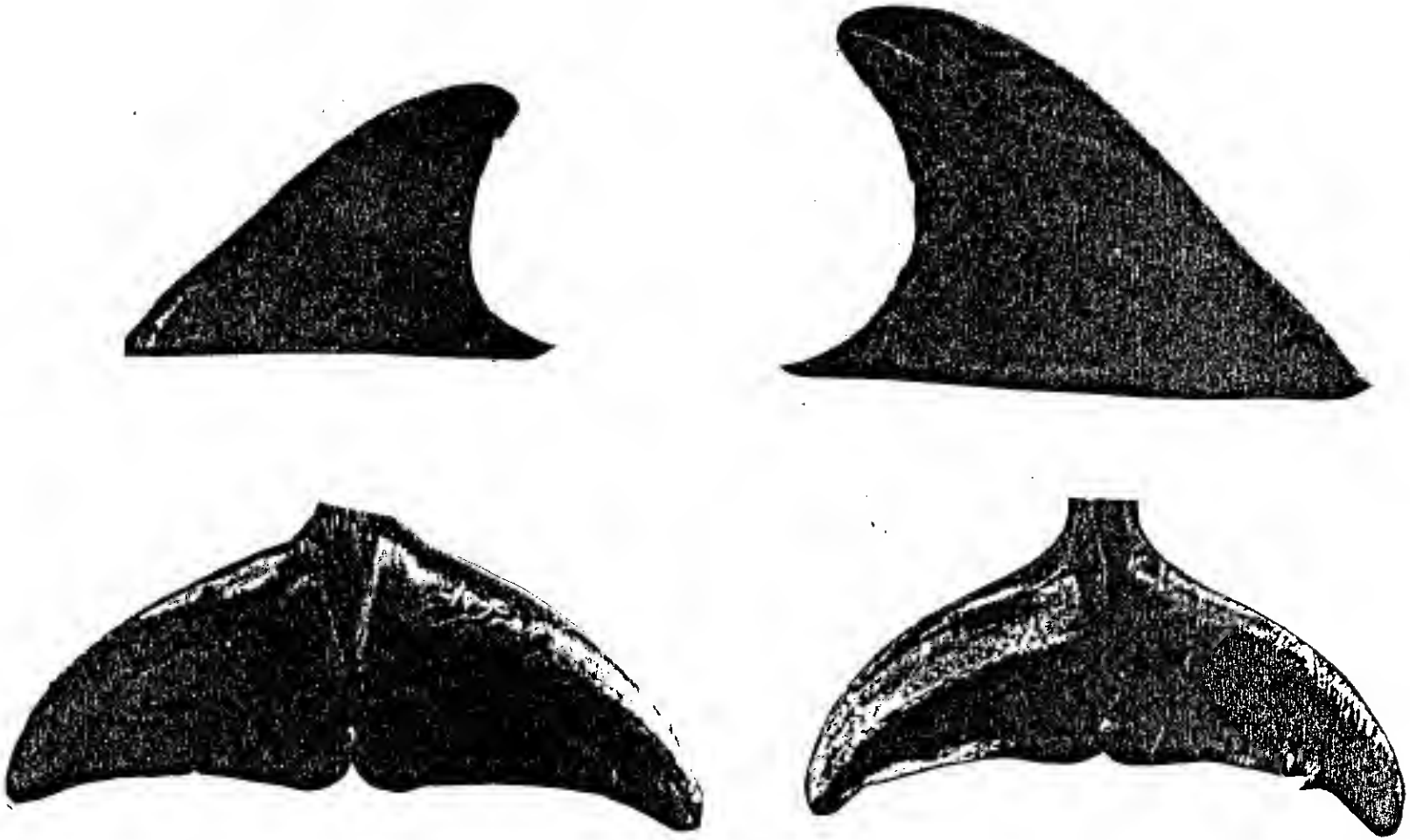

Figure 29. Photographic profile of dorsal fin and fluke of animal no. 646 on the left and animal no. 647 on the right.

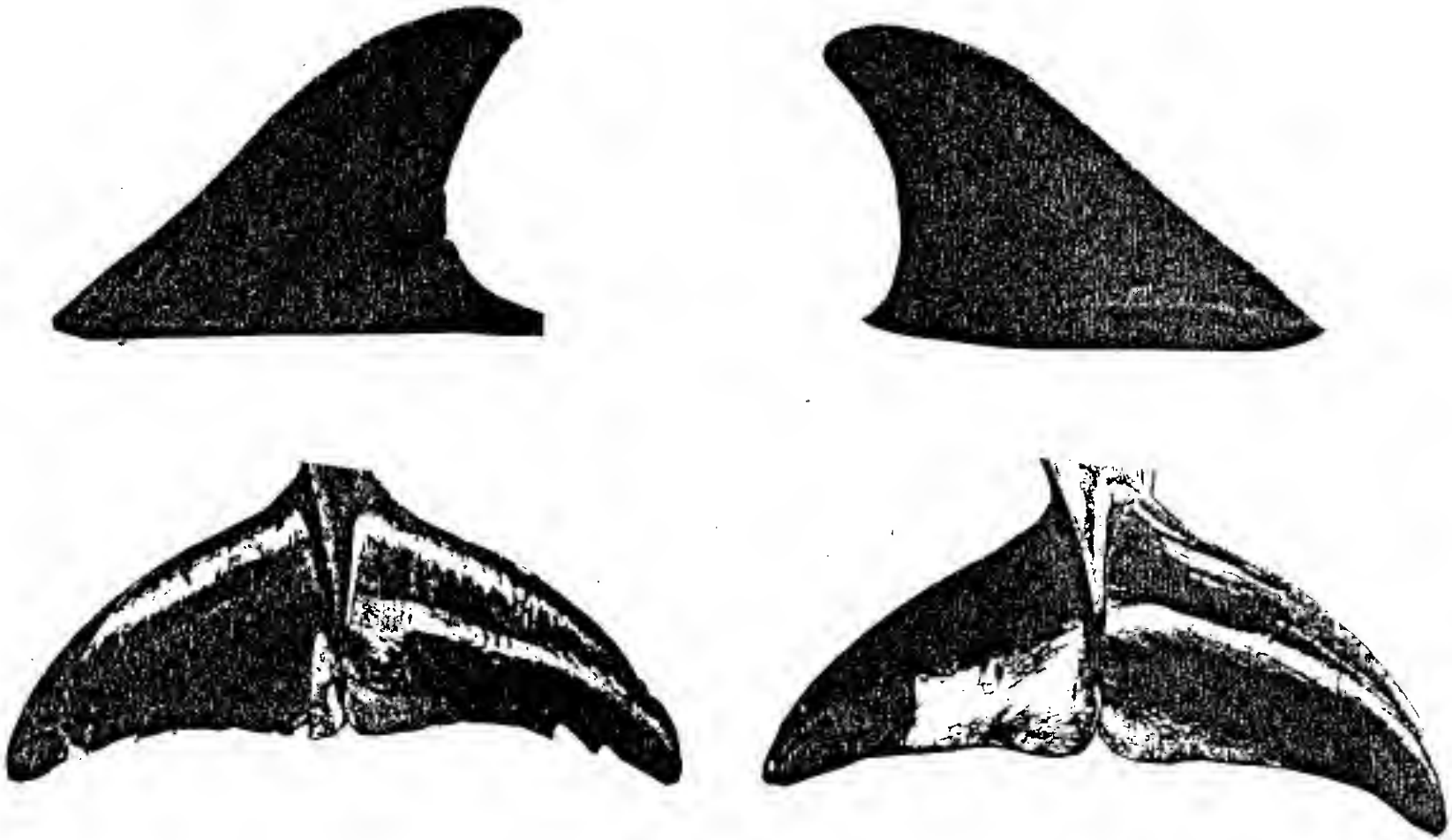

Figure 30. Photographic profile of dorsal fin and fluke of animal no. 648 on the left and animal no. 649 on the right.

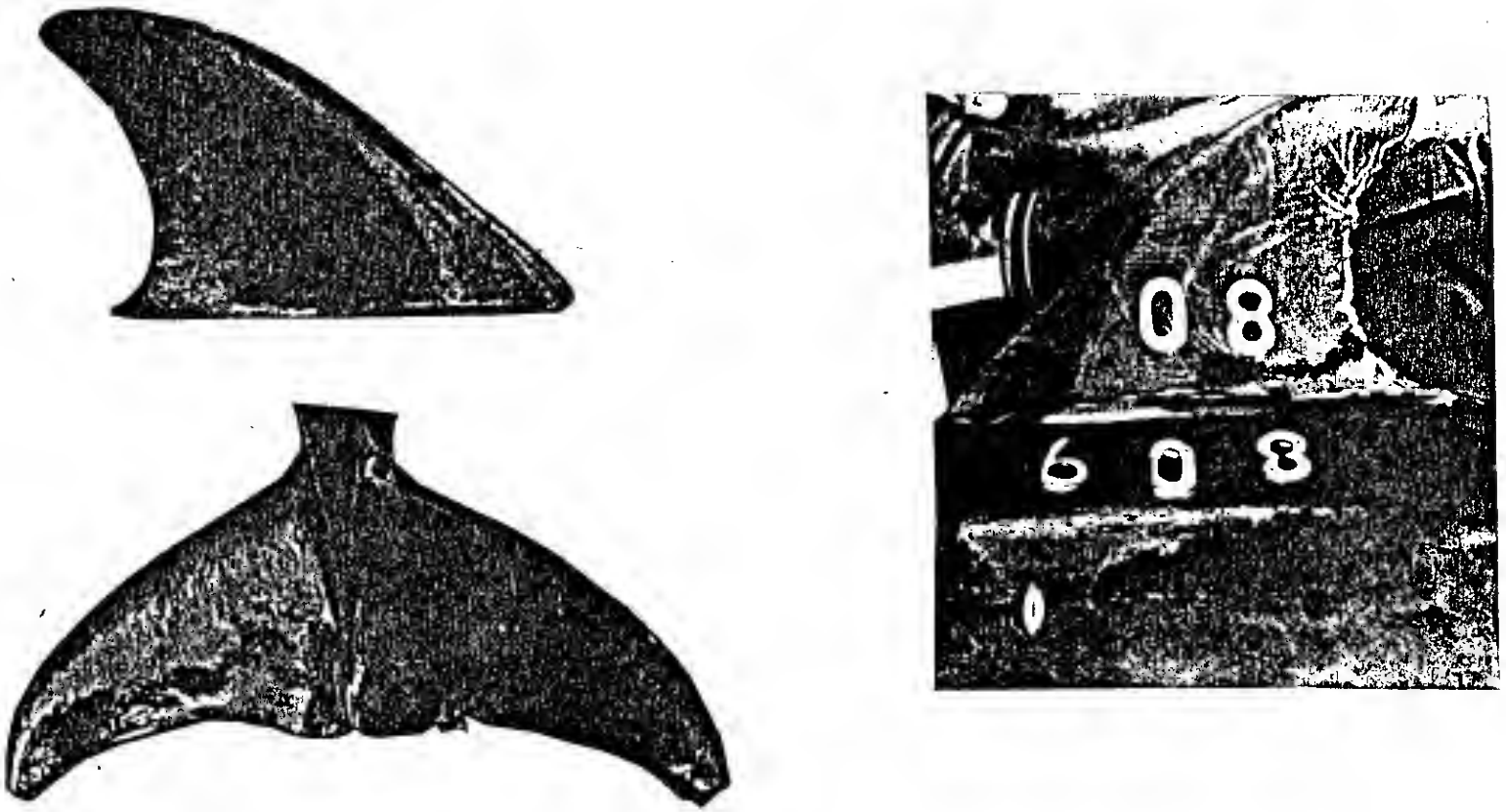

Figure 31. Photographic profile of dorsal fin and fluke of animal no. 650 on the left and the dorsal fin and side of animal no. 608 recaptured 21 days after processing on the right.

TISSUE BIOPSIES

Methods and Materials:

Skin, blubber, and liver biopsies were taken from several animals.

Skin and blubber samples were taken from the side of the animal with a specially designed tool (see Figure 1). Basically, it was a single-edge razor blade fitted on the posterior end of a 3cc syringe. Samples were obtained by simply gouging 10 to 13 mm of skin and blubber. This proved to be the most efficient method for obtaining skin/blubber samples. Five millimeter square pieces of skin were preserved in 10% neutral buffered formalin. Each blubber tissue sample was approximately 5 to 7 cm long and 10 to 13 mm deep. In most cases it took 2 to 4 seconds to obtain a blubber/skin sample. Blubber tissue was wrapped and sealed in aluminum foil and then placed immediately for one to two minutes in liquid nitrogen for quick freezing. Liver biopsies were obtained with the aid of a 15 cm long, Tru-Cut human biopsy needle (Travenol Laboratories, Inc., Deerfield, Illinois). Liver tissue samples measured approximately 15 mm long and 1 mm in diameter. Each tissue sample was divided into two pieces. Once piece was fixed in 3% Glutaraldehyde and processed for electron microscopy and the other half in 10% neutral buffered formalin for histopathology.

Results and Discussion

A total of 17 liver biopsies (9 males and 8 females) and 28 skin-blubber biopsies (14 males and 14 females) were collected. Table 1 shows the number, size, sex, age of biopsied animals. All blubber samples are being maintained in the freezer and awaiting processing when funding becomes available. The liver tissue has been processed to the pellet stage for electron microscopy, while the skin tissue is being preserved in 10% formalin. These will be analyzed when funding becomes available.

TABLE 1. SKIN, BLUBBER, AND LIVER BIOPSIES OF BOTTLENOSE DOLPHINS SAMPLED FROM THE MISSISSIPPI SOUND.

BRAND NO.	LENGTH	WEIGHT	SEX	AGE	LIVER	SKIN	BLUBBER
601	247	140	F	16	-	+	+
602	221	136	F	9	-	+	+
603	208	104	M	3	-	+	+
604	208	100	M	3	-	+	+
605	244	149	F	9	-	+	+
606	212	104	F	5	-	+	+
607	207	111	M	5	-	+	+
608	247	159	M	9	-	+	+
609	214	131	M	8	-	+	+
610	246	168	F	12	-	+	+
611	237	152	F	8	+	+	+
612	233	170	M	22	+	+	+
613	255	222	M	12	+	+	+
616	216	111	F	6	-	+	+
617	249	140	F	Not Determined	+	+	+
618	232	161	F	14	+	+	+
621	214	127	M	4	+	+	+
624	239	152	M	7	+	+	+
630	232	143	M	4	+	+	+
633	246	204	M	16	+	+	+
635	257	220	M	Not Determined	+	+	+
638	254	193	F	6	+	+	+
642	222	131	M	8	+	+	+
643	221	136	M	8	+	+	+
644	232	152	F	15	+	+	+
645	239	177	F	21	+	+	+
646	230	145	F	8	+	+	+
648	232	170	F	5	+	+	+
				Total	17	28	28
				Males	9	14	14
				Females	8	14	14

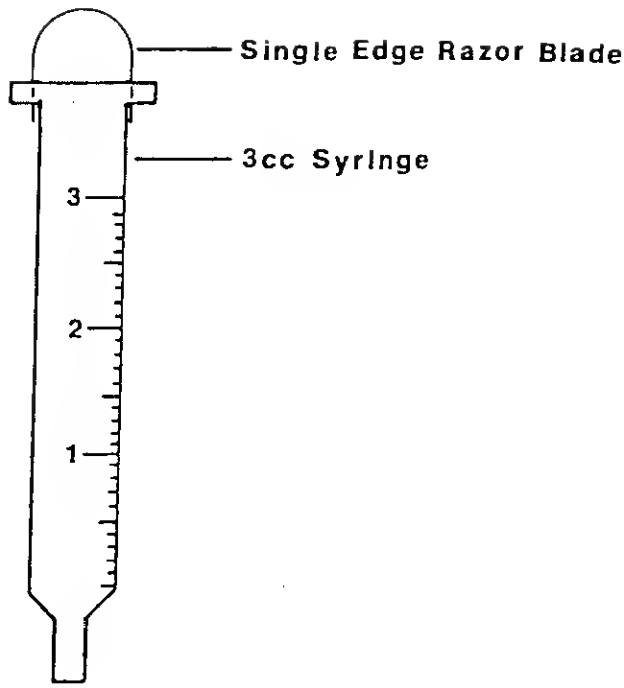

Figure 1: Tool used to gouge skin/blubber samples from dolphins.

MICROBIOLOGICAL PROFILES OF THE ATLANTIC BOTTLENOSE DOLPHINS, *Tursiops truncatus*, FROM THE MISSISSIPPI SOUND

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I. Introduction

From the 50 dolphins sampled, a total of approximately 1000 microbial isolates were selected and attempts made to identify them. The amount of funds available made it necessary at the outset to place limitations on the range of isolation media employed. In particular, it was necessary to either use standard isolation media employed for medical microbiology or to use media especially designed for isolation of marine microbes. It was decided to use standard media, since the availability of micro-test kits for identification of organisms isolated by this media would make it possible to identify a large number of isolates and because similar media were employed in the Indian River study. A detailed description of the isolation and identification procedures follows.

II. Materials and Methods

Collection of Samples. Samples were taken in the field from several sites on each animal (blowhole, anus, blood, vagina). Blood samples were drawn by syringe and immediately inoculated into blood culture bottles for transport and incubation. All other samples were taken using the culturette swab system, in which the area to be sampled is swabbed, then the swab is placed into a transport tube containing Modified Stuart's Transport Medium. All samples were kept on ice pending transport to this laboratory (always within 24 hours). Upon reaching the laboratory blood culture bottles were placed at 37°C for incubation and all other samples were inoculated onto

isolation media described below.

Isolation media. Selection of isolation media depended on the anatomical sampling site. Media utilized by site were as follows:

a) Anus:

5% sheep blood agar plate
MacConkey agar plate
Blood azide agar plate (starting with Dolphin #629)
Mycosel agar plate (or slant)
Tetrathionate broth tube

b) Blowhole:

5% sheep blood agar plate
MacConkey agar plate
Blood azide agar plate (starting with Dolphin #629)
Mycosel agar plate (or slant)
Tetrationate broth tube
5% sheep blood agar plate, incubated in candle jar (reduced O_2 tension)

c) Vaginal:

5% sheep blood agar plate
MacConkey agar plate
Blood azide agar plate (starting with Dolphin #629)
Mycosel agar plate (or slant)
5% sheep blood agar plate, incubated in candle jar (reduced O_2 tension)

d) Blood (only positive blood culture tubes were inoculated onto solid media)

5% sheep blood agar plate
Mycophil agar plate
Blood azide plate

Representative bacterial colonies were picked off all isolation plates and inoculated onto BHI slants for maintenance. Twenty-four hour old slant cultures were used for performing gram stains. All gram negative rods not isolated on MacConkey agar were inoculated thereon to determine ability to grow. A catalase test was performed on all bacterial isolates. An oxidase test was performed on all gram negative

organisms, and an OF-glucose test was performed on all gram negative organisms. On the basis of these reactions, all isolates were divided into groups for identification of schema. The groups are as follows.

1. Enterobacteriaceae (gram-rods, fermentative based on OF glucose oxidase negative, growth on MacConkey). Subsequent identification of this group was by the Minitek Enteriobacteriaceae system.

2. Oxidizers and oxidase + fermenters (gram-rods, oxidative by O-F glucose, oxidase +, growth on MacConkey). Subsequent identification of this group was by the schema presented in Biochemical Tests for Identification of Medical Bacteria (Jean F. McFaddin, Williams, ad Wilkins Co., Baltimore 1976) and by appropriate reference to Bergey's Manual of Determiniative Bacteriology (8th Edition). Primary criteria included cellular morphology; pigmentation; mobility, lysine, ornithine and arginine metabolism. Other biochemical tests were employed as required according to the schema.

3. Non-utilizers (gram-rods, carbohydrate inert by OF glucose). Subsequent identification of this group was by the Minitek Non-utilizer system.

4. Staphylococus/Micrococcus and related forms (gram + cocci, catalase +). Subsequent identification of this group was by the schema presented by Biochemical Tests for Identification of Medical Bacteria. Primary criteria included O-F glucose reaction, ability to grow on mannitol salt, coagulase reaction and pigmentation. Other biochemical reactions were employed where necessary.

5. Streptococcus (gram + cocci, catalase-). Subsequent identification of this group was by the schema presented in Biochemical Tests for Identification of Medical Bacteria. Primary criteria included hemolysis type on blood agar, optochin sensitivity, bile solubility bacitracin sensitivity, growth at 45°C and growth in 40% Bile.

6. Gram + rods, catalase +. Subsequent identification of this group was by the schema presented in the Biochemical Tests for Identification of Medical Bacteria. Primary criteria included spore function (determined by spore stain), arrangement of cells, O-F glucose reaction and motility.

7. Gram + rods, catalase-. Subsequent identification of this group was by the schema presented in Biochemical Tests for Identification of Medical Bacteria. Primary criteria included spore formation, O-F glucose reaction and production of H_2S.

8. Gram - cocci. Subsequent identification was based on O-F glucose reaction, motility, oxidase production and phenylalanine breakdown (all according to the schema of Biochemical Tests for Identification of Medical Bacteria) and on the Minitek Neisseriaceae System.

Colonies isolated on Mycosel or Mycophil agar were separated according to whether they were obviously molds (presence of mycelial mat) or whether they were yeasts or bacteria (colony morphology and cellular morphology upon gram stain). Identification of molds was by a standard taxonomic key and identification of yeasts by the Minitek yeast system. Bacteria isolated from Mycosel or Mycophil agar were grouped and identified as described above.

Results: Altogether approximately 1000 colonies were selected for identification (including approximately 150 selected from mycosel or mycophil media). In selecting colonies we attempted to choose at

least one colony of each colonial morphology type observed. Usually, several colonies of each type observed were picked since of course many species of bacteria produce colonies of similar appearance. All isolates were given a code number showing the animal from which the isolate was taken, the anatomical site, and the medium used for isolation. The code system used is described in Appendix A. A number of the colonies selected could not be successfully transferred or failed after one transfer, possibly due to some special growth requirement not provided in our isolation system. Nevertheless, at least 43 different species of bacteria were isolated. Table 1 summarizes the results, showing the organisms identified from each dolphin by site. As may be seen from the table, some sites from some animals have no identified isolates listed (e.g. dolphin 610, anus). There are several explanations for this. In some cases, no colonies were found on the isolation media. This happened only when the swabs were dry, that is when the collector failed to crush the transport medium vial to wet the swab. In some cases, a number of colonies were picked from the isolation media, but they could not be successfully transferred and were lost. The majority of cases is due to inability to identify certain isolates by the schema employed. Only those isolates which fit identification schema are shown on the table. Where the organism could only be identified to the genus level, it was generally because at least one of the tests for identification yielded results atypical for any of the species usually identifiable by the system used, but the remaining results were compatible with the genus proposed.

TABLE 1: Results of bacterial cultures from 50 bottlenose dolphins.

SITE: Anus

Dolphin Number and Sex

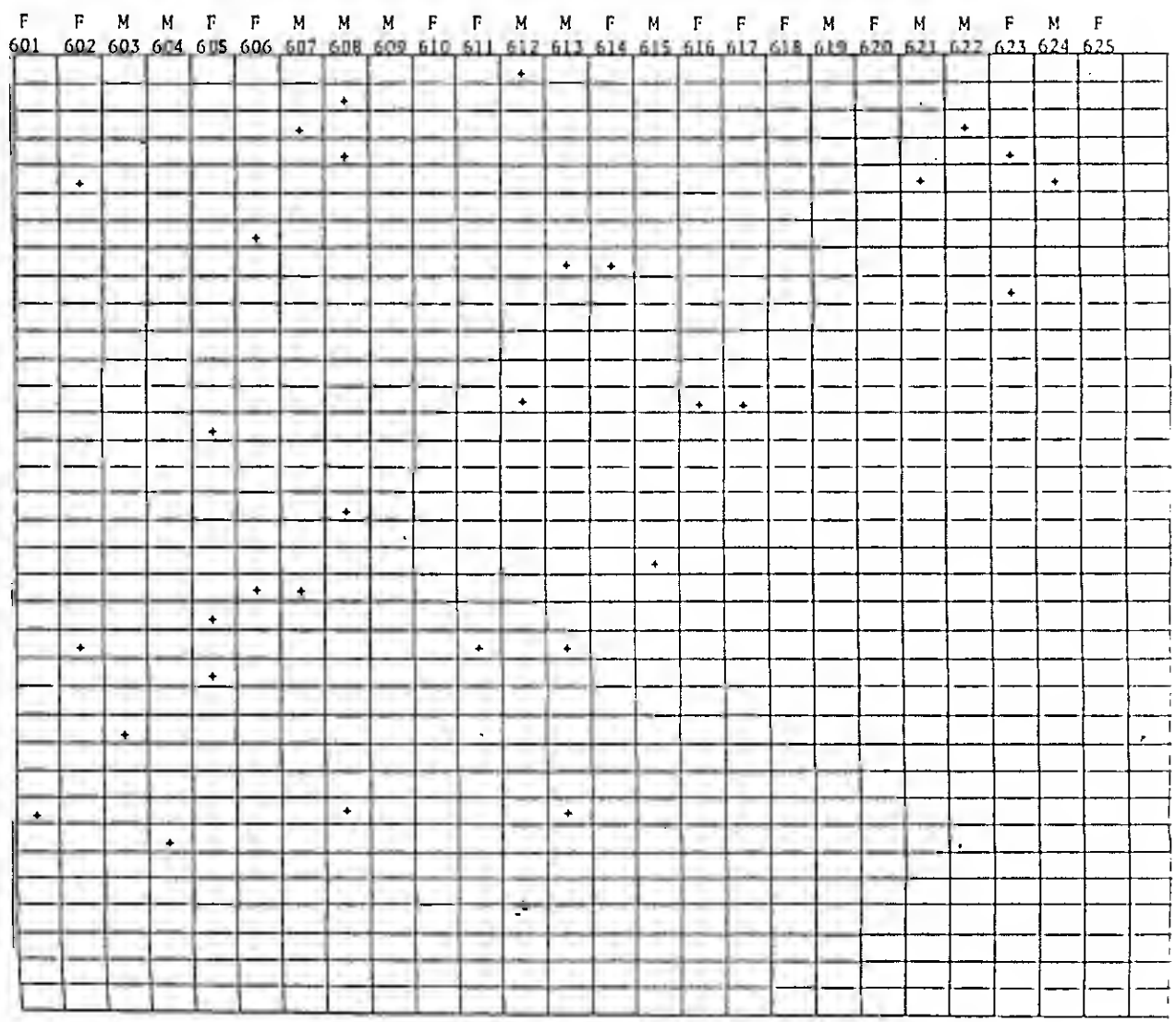


TABLE 1: Results of bacterial cultures from 50 bottlenose dolphins.

SITE: Anus

Dolphin Number and Sex

Organism	F 626	F 627	F 628	F 629	M 630	M 631	F 632	M 633	F 634	M 635	M 636	F 637	F 638	F 639	F 640	F 641	M 642	M 643	F 644	F 645	F 646	F 647	F 648	M 649	F 650	#
Achromobacter sp																										1
Acinetobacter sp																										1
A. anitratus																										2
A. lwoffi						+					+	+														5
Aeromonas sp											+									+						5
Bacillus cereus			+																							1
Branhamella catarrhalis																										1
CDC Group VE Biotype 1																										2
Citrobacter diversus																										1
C. freundii																								+		1
Edwardsiella sp																					+		+			2
Enterobacter agglomerans																					+					1
Escherichia coli								+				+	+	+		+		+		+		+	+			12
Flavobacterium tirrenicum				+																						2
Hafnia alvei				-																					+	1
Klebsiella ozaenae																					+					1
K. rhinoscleromatis							+							+												3
Moraxella sp								+																		1
Morganella morganii												·														1
Neisseria flavescens																										2
Pasteurella sp																										1
Pseudomonas sp								+	+			+					+									7
P. paucimobilis																										1
P. pseudoalcaligenes					+			+																		2
P. putrifaciens																										1
Proteus sp	+		+				+																			3
P. mirabilis							+																			1
Shigella sp			+			+						+													+	7
Staphylococcus sp																						+				1
Vibrio sp			+			+																				2
Yersinia "enterocolitica"(GP)												·	+													1

TABLE 1: Continued

SITE: Blowhole

Dolphin Number and Sex

Organism	F 601	F 602	M 603	M 604	F 605	F 606	M 607	M 608	M 609	F 610	F 611	M 612	M 613	F 614	M 615	F 616	F 617	F 618	M 619	F 620	M 621	M 622	F 623	M 624	F 625
Acinetobacter sp									+						+										
A. lwoffi				+			+		+																
Aeromonas sp																									
Bacillus cereus																									
Branhamella catarrhalis																+									
CDC Group VE Biotype I				-							+			+											
Citrobacter freundii																									
Edwardsiella tarda	+											+													
Enterobacter agglomerans																									
Escherichia coli					+			+																	
Flavobacterium indoltheticum			+																						
F. tirrenicum																+									
Hafnia alvei																									
Micrococcus sp		+																			+				
Moraxella sp			+		+								+												
Neisseria subflava			+																						
Planococcus sp		+		+			+																		+
Pseudomonas sp							+	+		+					+										
P. pseudoalcaligenes					+																				
P. putrifaciens				-	+	+																			
P. stutzeri													+												
Salmonella enteriditus														+											
Shigella sp				+				+																	
Staphylococcus sp																+									
Vibrio sp	+																								
Yersinia "enterocolitica"(GP)																									

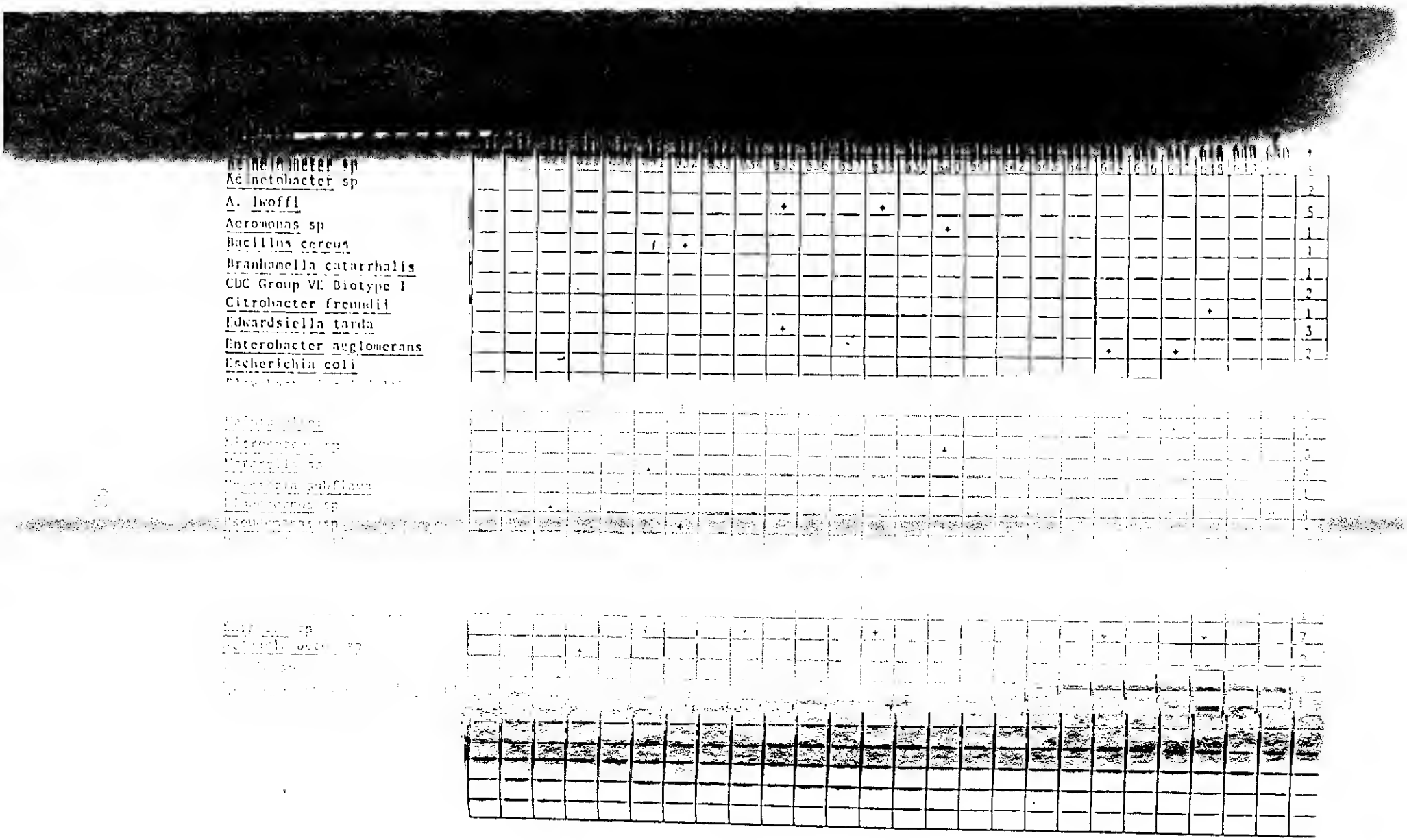

Acinetobacter sp
A. lwoffi
Aeromonas sp
Bacillus cereus
Branhamella catarrhalis
CDC Group VE Biotype 1
Citrobacter freundii
Edwardsiella tarda
Enterobacter agglomerans
Escherichia coli

TABLE 1: Continued

SITE: Vagina

Dolphin Number and Sex:

Organism	F 601	F 602	M 602	M 604	F 605	F 606	M 607	M 608	M 609	F 610	M 611	M 612	M 613	F 614	M 615	F 616	F 617	F 618	M 619	F 620	M 621	M 622	F 623	M 624	F 625
Acinetobacter sp						+																			
A. lwoffi						+				+															
Aeromonas sp																									+
Bacillus subtilis					+																				
Branhamella catarrhalis											+														
CDC Group VE Biotype I																									
Citrobacter amolonaticus																									
C. freundii																									
Enterobacter agglomerans																	+								
Escherichia coli	+													+											
Flavobacterium indoltheticum																							+		
Klebsiella sp		+																							
Micrococcus sp	+																								
Moraxella sp																									
Moraxella/Pasteurella sp					+																				
Neisseria flavescens																									
Proteus morganii	+	+			+	+				+										+					
Pseudomonas sp																									
P. diminuta		+																					+		
P. putrifaciens																									
Shigella sp	+																								
Staphylococcus sp																									+
Vibrio sp																									
V. anguillarum																									
SITE: Blood																									
Bacillus subtilis												+										•			
Pseudomonas sp						+																			

TABLE 1: Continued

SITE: Vagina

Dolphin Number and Sex

Organism	F 626	F 627	F 628	F 629	M 630	M 631	F 632	M 633	F 634	M 635	M 636	F 637	F 638	F 639	F 640	F 641	M 642	M 643	F 644	F 645	F 646	F 647	F 648	M 649	F 650	#
Acinetobacter sp																										1
A. lwoffi											+															3
Aeromonas sp				+							+															2
Bacillus subtilis																										1
Branhamella catarrhalis																										1
CDC Group VE Biotype I																										1
Citrobacter amolonaticus																+										1
C. freundii																									+	1
Enterobacter agglomerans																						+				1
Escherichia coli																+						+				3
Flavobacterium indoltheticum																										2
Klebsiella sp																										1
Micrococcus sp																										1
Moraxella sp																										1
Moraxella/Pasteurella sp							+																			1
Neisseria flavescens																										1
Proteus morganii															+											1
Pseudomonas sp							+																			7
P. diminuta									+																	1
P. putrifaciens													+													2
Shigella sp																										1
Staphylococcus sp	+																									2
Vibrio sp													+													1
V. anguillarum														+											.	1
SITE: Blood																										
Bacillus subtilis																						.				1
Pseudomonas sp													.													1

Organisms identified by one of the Minitek systems frequently were given probabilities of identity in the Minitek code books. If the organism fit one of the possible codes exactly and if the probability of identity for the most likely organism was at least 80%, that organism is the one listed in Table 1. If the most likely probability of identity was less than 80% or if the code did not fit any of the possible organisms even at the genus level, the identity of the isolates was considered too uncertain to include Table 1. Table 2 contains those uncertain organisms and shows the maximum probabilities of their identities. Keep in mind that where the listed probability of identity is greater than 80% (e.g. isolate 614CB1 is listed as 98.13% certain to be compatible with CDC group VE, Biotype 1), the code did not completely fit the Minitek code, or the organism would not be listed in Table 2. Thus at least one identification reaction was incompatible with the Minitek identification scheme, and the actual probability of identification must be considered to be much lower.

Identification of molds and yeasts is nearly complete. A complete list of fungal isolates from Mycosel or Mycophil agar is found on Table 3, with tentative identification where available. Final identification of yeasts is awaiting arrival of special carbohydrate utilization media. Molds tentatively identified to genus level do not completely fit any species, but continued efforts are being made to determine species. The unidentified molds are very unusual organisms which have so far resisted our efforts to key them. A number of yeast isolates have been tentatively identified as Saccharomyces,

TABLE 2: Uncertain Isolates

BLOWHOLE

Dolphin #	Isolate #	Probability of Identity		
611	BB1	CDC group VE Biotype I 75.73%	Pasteurella haemolytica 24.28%	Haemophilus aphrophilus 0.02%
613	CB1	Moraxella sp. 78.99%	Alcaligenes sp 17.45%	Pseudomonas diminuta 2.89%
614	CB1	CDC group VE Biotype I 98.13 %	Acinetobacter anitratus 0.95%	Pasteurella haemolytica 0.87%
614	BB1	CDC group VE Biotype I 98.13%	Acinetobacter anitratus 0.95%	Pasteurella haemolytica 0.87%
631	CB2	Moraxella sp 78.99	Alcaligenes sp 17.45%	Pseudomonas diminuta 2.89%
634	BB1	Alcaligenes/ Pseudomonas sp 46.91%	Acinetobacter lwoffi 33.31%	Bordetella parapertussis 8.93

ANUS

Dolphin #	Isolate #	Probability of Identity	
650	MA1	Enterobacter agglomerans 60.79%	Providencia stuartii 35.6%

VAGINA

Dolphin #	Isolate #	Probability of Identity		
611	CV1	CDC group VE Biotype I 98.13%	Acinetobacter anitratus 0.95%	Pasteurella haemolytica 0.87%
632	MV2	Moraxella sp 78.99%	Alcaligenes/ Pseudomonas sp 17.45%	Pseudomonas diminuta 2.89%

TABLE 3: Yeast & Molds isolated from Mycosel or Mycophil with tentative identification.

Site	Isolate #	Tentative identity
Blowhole	616YB1	Unidentified yeast (*Saccharomyces* sp)
	619YB1	Unidentified yeast (*Saccharomyces* sp)
	625YB1	Unidentified mold
	634YB1	Unidentified mold
	636YB1	*Mucor* sp
	640YB1	Unidentified yeast (*Saccharomyces* sp)
	640YB2	Unidentified mold
	650YB1	*Penicillium* sp
Anus	607YA1	*Rhodotorula* sp (pos *rubra*)
	650YA1	Unidentified yeast (*Saccharomyces* sp)
Vagina	625YV1	*Aspergillus* sp
	629YV1	Unidentified yeast (*Saccharomyces* sp)
	638YV1	Unidentified mold

including 5 from Table 3 and a number isolated from bacteriological isolation media. Molds (or yeasts) which ultimately cannot be identified will be sent to a mycological laboratory for identification.

Several bacterial "isolates" have been of interest in that they are composed of apparently two very different (morphological) organisms. These "isolates" have resisted all efforts to obtain pure culture and cannot be identified by any system we have employed. It may be that these represent two organisms, have a mutual dependence, or we may be dealing with a very pleormorphic organism for which we have no suitable identification system.

Discussion: We found few surprises in the results obtained. It is of interest that we find a somewhat different array of organisms than observed in the Indian River study. Most of the organisms which we found are consistent with what one would expect in marine mammals from Gulf Waters. It should be pointed out that codified identification systems such as the Minitek are designed for certain groups of organisms likely to be encountered under a particular situation. The Minitek system is primarily designed to identify bacteria of medical importance from clinical samples. Since we were dealing with marine mammals, it is quite likely that the flora we were dealing with included organisms for which the Minitek system was not designed and which would produce a code not precisely fitting any of the organisms listed in the Minitek identification book. There are several ways to approach this problem. One can use a complicated taxonomic key approach to identification, which is effective but costly in time and money. We plan to pursue such an approach with a limited number of the organisms

we isolated during the course of the study and we will furnish a supplemental report indicating the results of this additional study. One can simply choose the best fit of the codes listed with the codes generated. This is often very practical when only one or two minor discrepancies exist between the generated code number and an actual code number, and it is the approach used to tentatively identify some of the organisms isolated here. A third approach is to simply identify the organism by the code number generated, and by various important morphological, cultural or biochemical traits observed. This approach is of particular value in comparing dolphins from different sites or captured at different times. We have of course recorded Minitek code numbers of all isolates tested and these numbers could be used for comparison in the event a second round of samping is contemplated.

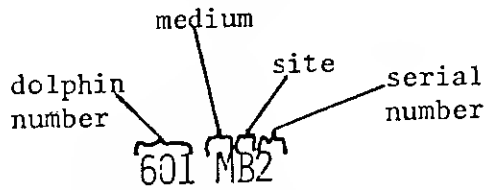
We will continue efforts to confirm identity of all isolates to at least the genus level and will include any forthcoming information in a supplemental report.

APPENDIX A. Code for identifying isolates.

For organizational purposes, each isolate was given a six or seven digit code identifying the animal from which it came, the media used to isolate it and the anatomical site from which it was taken. The code consists of three digits identifying the dolphin number, followed by a single letter identifying the media used, followed by a single letter identifying the anatomical site, followed by one or two digits providing a serial number of the isolate. A summary of the code symbols follows.

	Dolphin number: 601, 602, . . . 650
Medium from which isolate was picked:	M(=MacConkey agar)
	B(=5% Sheep blood agar)
	C(=5% Sheep blood agar under CO_2)
	Y(=Mycosel or mycophil agar)
	T(=Tetrathionate broth)
	A(=5% Sheep blood agar with sodium azide)
Anatomical site	B(=Blowhole)
	V(=Vagina)
	A(=Anus)
	S(=Blood)
	Isolate serial number: 1, 2, . . . n (depending on number picked from the particular medium)

The following is an example of a typical code



This isolate was taken from the blowhole of dolphin number 601 using MacConkey agar. It was the second such isolate picked.

BIOCHEMICAL GENETICS

Serum Protein and Hemoglobin Electrophoretic Profiles In *Tursiops* from the Northern Gulf

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I. INTRODUCTION

It is well accepted that the enzyme and protein profile of an organism is a direct reflection of its gene composition. While the protein/enzyme makeup of individuals within a species is very similar, non-lethal mutations or gene changes which are then replicated and passed on to progeny in subsequent generations result in inherited differences between individual members of the species which are normally expressed as normal individual, physiological and mental characteristics.

The careful selection of proteins/isoenzymes for characterization phenotyping permits one to study the gene makeup and/or variability of given populations. Such studies are important in following genetic differences between "social units" of species such as Tursiops, as well as assigning mother-calf and father-calf pairs within a social unit.

II. MATERIALS AND METHODS

Rationale for selection of techniques

The complete characterization of hemoglobin phenotypes, plasma proteins and plasma/RBC isoenzymes requires a variety of characterization techniques. While electrophoretic separations play an important role in all such characterizations, any conclusions drawn from such preliminary studies must be confirmed by additional techniques. Because of the time and cost restraints of this project, such complete phenotype characterization was not possible. Rather, methods were selected which

would yield the most information in a timely, cost efficient manner. The methods chosen and the rational for these selections follow.

Complete phenotyping of hemoglobin involves:(1) initial electrophoretic screening on polyacetate. (2) Those samples suggesting abnormal patterns are then subjected to two or more additional electrophoretic separations employing various buffers and pH's to establish a preliminary Hemoglobin phenotype. (3) Hemoglobin chains are then separated and subjected to various chromatographic techniques to isolate individual chains. (4) Isolated chains are then analysed to determine which chain contains the abnormality and often subjected to peptide fingerprinting to completely characterize the abnormality. For this study, in order to obtain the most information on each hemoglobin sample in a timely manner, we selected isoelectric focusing as a one step characterization step. Although both more costly and more technically demanding than other electrophoresis methods, abnormal hemoglobins are readily identified, and even tentative assignments to both chain substitution and types of substitution can be made. Thus, we selected this one technique to replace the normal 3-4 electrophoretic separations and column chromatography steps normally required to detect hemoglobin abnormalities, and to assign tentative biochemical/genetic phenotypes for these variable hemoglobins.

Isoenzymes require electrophoretic separations followed by kinetic and/or stability studies in order to completely phenotype a given enzyme. By using the resolving power of polyacrylamide electrophoresis in place

of starch or polyacetate electrophoresis, we were able to phenotype a number of enzymes following electrophoresis. In addition, the slabs of polyacrylamide were readily dried and preserved for possible future interpretations.

Plasma electrophoresis involves the separation and identification of a large number of different proteins. Since special stains, electrophoretic conditions, etc. are required for each protein, a complete characterization of all plasma proteins was clearly beyond the scope of this project. Rather than to select 2 or 3 of these proteins for such detailed characterization, we subjected all samples to a preliminary screening using agarose electrophoresis. It was the intent of this screening to identify those proteins and/or dolphin samples which would yield the greatest return on a more complete characterization in future projects of this type.

Collection and preparation of samples.

Blood samples were drawn in the field by syringe, placed into heparinized tubes, and kept on ice pending transport to the laboratory (always within 24 hours). Upon reaching the laboratory, the blood was centrifuged to separate the erythrocytes from the plasma. Immediately following centrifugation, 100 µl aliquots of plasma were placed in individual containers, quick frozen in liquid nitrogen, and maintained at -70°C pending analysis.

Erythrocytes were washed three times with normal saline, with a low speed centrifugation between each wash. Following the final wash, the erythrocytes were hemolyzed by the addition of an equal volume of distilled/deionized water. Aliquots of 100 µl were placed in individual

containers, quick frozen in liquid nitrogen, and maintained at -70°C pending analysis. To assure that these freezing/storage conditions did not lead to extraneous results and interpretations, fresh samples of both plasma and hemolysates were analyzed for each of the plasma proteins and hemolysate hemoglobins and isoenzymes discussed in this report. In each instance, the same results were obtained for the fresh and frozen samples, illustrating the validity of the storage techniques.

Plasma Protein Electrophoresis

Serum proteins were separated utilizing 1% agarose gels, pH 0.7, measuring 100 X 100 X 0.7 mm. Immediately following thawing, plasma was diluted 1:5 with distilled water containing Bromophenol blue added as tracking dye. One µl of each sample was then applied to the gel in duplicate, and the gels placed in a Bio-Rad model 1400 electrophoresis chamber. Voltage (90 volts) was applied for 2 hours, or until the tracking dye reached the end of the gel. Gels were then removed from the chamber, fixed in 1% picric acid for 10 minutes, washed with ethanol to remove excess picric acid, pressed between filter paper for 10 minutes and air dried at 70°C for an additional 10 minutes.

Dry gels were then stained with 0.1% Coomasie Brilliant Blue R250 for 30 minutes, destained with ethanol/acetic acid/water (25:8:65), and air dried at room temperature.

Hemoglobin isoelectric focusing

Hemoglobins were separated on horizontal polyacrylamide slabs using the technique of isoelectric focusing.

Gels, measuring 125 x 260 x 0.7 mm, and containing 6.5% acrylamide, 0.2% Bis acrylamide and 2.0% Biolyte (Bio-Rad Laboratories) were prepared fresh daily by polymerizing gels onto Gell Bond (Marine Colloids) in the presence of Riboflavin. To ensure complete polymerization, gels were placed in front of flourescent lights overnight. Following polymerization, 20 ul of sample (hemolysate) was applied and separation then carried out in a LKB, model 2117 multiphore electrophoresis chamber at 15 m amps. When 1000 volts was reached, the voltage was held constant until the visible, hemoglobin bands sharpened. The gel was then removed from the chamber, fixed in 5% trichloracetic acid for 5 minutes, and soaked in 5% acetic acid containing 1% glycerol overnight. Gels were then placed between 2 sheets of dialysis membrane and dried under vacuum on a Hoeffer, model SE 540 gel slab dryer.

Isoenzyme and Haptoglobin electrophoresis.

Electrophoretic separations were conducted on 6% - 8% step gradient polyacrylamide gel slabs measuring 70 x 80 x 3 mm. All separations employed a Tris-sulfate, tris-borate continuous buffer system. An Isolab, Uniscil vertical, gel slab electrophoresis unit was employed for all experiments, which were carried out at 4°C. Following application of 10 µl samples to the gel, pulsed current (50 to 200 pulses/sec at 1 µF discharge capacitance) was applied to the gels at a constant 280 volts until tracking dye (bromphenol blue) reached the bottom of the gel (approximately 2 hours). Gels were then removed from the electrophoresis chamber, sliced in half to expose an interior surface and stained.

The following solutions and conditions were used for detection of isoenzymes following electrophoretic separation.

1. Esterase D was detected by incubating the gels in 4-methylumbelliferyl acetate, pH 5.2. Zones of activity of Esterase D activity floureced when the gels were observed under u.v. light.
2. Glucose-6-phosphate dehydrogenase(G6PD) was stained by incubating gels with glucose-6-phosphate, NADP, $MgCl_2$, phenazine methosulfate (PMS) and nitroblue tetrazoleum (NBT), all dissolved in 0.5 M tris, pH 7.5. Zones of GPD activity appeared as dark blue bands.
3. Lactate Dehydrogenase (LDH) activity also appeared as blue bands when gels were incubated with sodium lactate, NAD, PMS, NBT and $MgCl_2$ in Glycine buffer, pH 9.5.
4. Malate dehydrogenase (MDH) isoenzymes were visualized by incubation of gels with malate, NAD, PMS, NBT and $MgCl_2$ at pH 7.0.
5. 6-Phosphogluconate Dehydrogenase was detected utilizing 6-phosphogluconate, NADP, PM5, $MgCl_2$ and NBT at pH 7.5.
6. Glyoxylase activity was visualized by incubation of the gels in glutathione, NBT, $MgCl_2$ and methylglyoxal dissolved in phosphate buffer; pH 6.7. These isoenzymes show up as clear zones on blue background.
7. Haptoglobin was detected by mixing 50 µl hemolysate with 400 µl plasma prior to electrophoresis. Following electrophoresis, haptoglobin was stained with benzidine and H_2O_2. Haptoglobin (and hemoglobin) first stain green, then form permanent brown zones.

Following staining, all gels were preserved by soaking in glycerol/acetic acid followed by dehydration as described previously. Unless

otherwise noted, all reagents for electrophoresis were purchased from Bio-Rad Laboratories. Substrates, dyes and coenzymes used in staining were all products of Sigma Chemical Co.

III. RESULTS AND DISCUSSION

A. Hemoglobin

Hemoglobin is the most widely studied and best understood of the many proteins which undergo genetic variation. Mammalian hemoglobin is a tetramer of four monomeric, polypeptide chains (subunits), two α chains and two non α chains. In adults, most of the hemoglobin is $\alpha_2\beta_2$ and is referred to as Hemoglobin A. In addition, $\alpha_2\gamma_2$ (Hb F) remains present throughout life (approximately 1% of total Hemoglobin) and $\alpha_2\delta_2$ (Hb A_2) makes up approximately 2% of the total hemoglobin.

As shown in Figure 1, when subjected to isoelectric focusing, the major components of human hemoglobin are readily separated into Hb A, Hb A_2, and Hb F. Also shown in Figure 1, is the hemoglobin profile of the 50 dolphin samples analyzed in this study. As can be noted from the figure, 47 of 50 samples exhibited patterns very similar to human hemoglobin. Actual measurement of the isoelectric point of the dolphin hemoglobins showed the dolphin hemoglobin components [Hb A, Hb A_2, Hb F] to be 0.1 pH higher than the corresponding human component.

Of the most interest to this study was the finding that three of the dolphins exhibited abnormal hemoglobin profiles. Dolphins number 636 and 640 exhibited identical profiles with three extra bands. As illustrated in the figure, these extra bands were associated with, and slightly higher than, each of the 3 hemoglobin components. This pattern is consistant

for a heterozygous individual with an amino acid substitution on the α chain. Since the α chain occurs in each of the hemoglobin components, one would get both a normal and an abnormal band for Hb A, Hb F, and Hb A_2. This is, of course, the situation for samples 636 and 640. Furthermore, since the isoelectric points of the abnormal bands are approximately 0.15 units higher than the normal component, it can be concluded that this amino acid substitution results in an increase of 1 charge per chain [i.e. a neutral amino acid for Asp or Glu; or Arg or Lys for a neutral amino acid]. Two common human hemoglobinopathies with such a substitution are Hb Montgomery (α-48 Arg for Leu) and Hb Russ (α-51 Arg for Gly). This conclusion regarding the chemical basis for these abnormalities is strengthened when the patterns for Hb Montgomery are compared to samples 636 and 640 (Figure 1).

Dolphin sample 609 exhibited a different abnormal hemoglobin profile. Specifically it was identical to the 47 "normal" dolphins except for the presence of an extra band approximately 0.15 pH unit above Hb A. This pattern is consistent with a heterozygote individual with an amino acid substitution on the β subunit. Since only Hb A contains a β subunit the only extra band would be on Hb A. Again, this increased P.I. of 0.15 units is suggestive of a substitution resulting in a net increase in charge of +1 [a neutral amino acid for Asp or Glu or Arg or Lys for a neutral Amino acid]. A number of such genetic variations occur in humans including Hemoglobin D-Punjab (β-121 Gln for Glu) and Hemoglobin Korle Bu (β-73 Asn for Asp). This is also the amino acid substitution which

results in sickle cell trait (Hb S) in humans (β-6-Val for Glu). This similarity between a heterozygous individual for sickle cell and Dolphin sample 609 is also illustrated in Figure 1.

Thus, it can be concluded that three of the fifty dolphin samples contain an abnormal hemoglobin. Based on results obtained to date, it would appear that number 636 and 640 are heterzygous with an amino acid substitution in the α chain producing a Hb Montgomery electrophoretic pattern. Sample 609 appears to be heterozygous with a substitution in the β chain producing a sickle cell type pattern. Clearly additional structural studies must be undertaken to both substantiate these conclusions and to determine whether these substitutions are in a position in the hemoglobin molecule which will effect the ability of hemoglobin to transport O_2.

B. Lactate dehydrogenase

One of the most studied of all the isoenzymes is lactate dehydrogenase (LDH). This isoenzyme is a tetramer composed of two different subunits (A & B) giving rise to 5 isoenzyme forms (A_4; A_3B; A_2B_2; AB_3; and B_4). In erythrocytes, all but the B_4(LDH_5 or slow isoenzyme) can be detected following electrophoresis.

In addition to these 5 isoenzymes, sub-banding is frequently encountered. Such sub-banding can best be explained in erythrocytes if, in addition to the normal B subunit, an abnormal B subunit is also present. In such a situation LDH 1 would exhibit only 1 band (A_4), LDH 2 would exhibit 2 sub-bands (A_3B and A_3B^*),LDH 3 would exhibit 3 sub-bands (A_2B_2; A_2BB^*; $A_2B_2^*$) and LDH 4 would exhibit 4 sub-bands(AB_3;AB_2B^*; ABB_2^*; and AB_3^*).

Alternatively, LDH could have a normal B subunit, but an abnormal A and normal A subunit. In this situation, LDH 1 would exhibit 5 sub-bands, LDH 2 would exhibit 4 sub-bands, LDH 3 would exhibit 3 sub-bands, LDH 4 would have 2 sub-bands and LDH 5 could have only 1 sub-band.

As shown in Figure 2, all dolphin hemolysates examined exhibited a banding/sub-banding pattern consistant with a single A subunit but containing 2 different B Subunits. Although four sub-bands would be expected in such a situation for LDH-4, the small amount of LDH 4 present in erythrocytes is not sufficient to detect these sub-bands, but rather, activity appears as a weak, diffuse zone over the LDH 4 region.

C. Glucose-6-phosphate Dehydrogenase (G6PD)

This enzyme catalyzes the oxidation of Glucose-6-phosphate to 6-phosphogluconate with a simultaneous production of NADH. This enzyme thus plays an essential role in the intracellulur NADH concentration. In humans, over 100 variants of G6PD have been reported. Such variants have received considerable attention because of the adverse clinical implication (hemolytic anemia) which frequently result from these variants.

G6PD is a hexamer of identical subunits. The locus for this enzyme is on the X chromosome, although in females, only one of the 2 alleles are functioning at the same time. Thus, both males and females exhibit only one form of the enzyme [no heterozygotes] and variants are detected electrophoretically as either a faster or slower moving variant.

Of the 50 dolphin hemolysates analyzed, only 1 variant, an abnormally slow electrophoretic pattern was found (Figure 3). However, it should be noted that this sample (636) was one of the 3 exhibiting an abnormal hemoglobin pattern.

D. Esterase D.

Esterase D (ESD) is one of four non-specific red cell esterases. It is detected, following electrophoresis, based on its ability to hydrolyze 4-methyl-umbelliferone. Three common phenotypes - ESD 1,ESD 2-1, and ESD 2 have been found in humans. As shown in Figure 4, all three phenotypes were found in the dolphin samples analyzed, with ESD 1 being found in 62% of the samples tested,ESD 2-1 in 30%, and ESD 2 in 8%.

E. 6-Phosphogluconate Dehydrogenase

Phosphogluconate dehydrogenase is a dimer which is controlled by 2, co-dominant alleles on the same loci. Three common phenotypes of this enzyme A, C, and AC ($PGD^{\alpha}PGD^{\alpha}$; $PGD^{\gamma}PGD^{\gamma}$, and $PGD^{\alpha}PGD^{\gamma}$) are found in human populations. The most common isoenzyme phenotype is the A form, which occurs in over 90% of the human population. In addition, other heterozygous conditions exist which result in a partial, quantitative deficiency of this enzyme.

As shown in Figure 5, only the common A phenotype ($PGD^{\alpha}PGD^{\alpha}$) was detected in the dolphin hemolosates examined in this study.

F. Malate Dehydrogenase

Malate dehydrogenase (MDH) catalyzes the reversible conversion of malate to oxaloacetate using NAD as the coenzyme. One form (s-MDH) is found in the cytoplasm, while a second (m-MDH) is found in mitochondria. Only s-MDH is found in erythrocytes.

A number of genetic variants of s-MDH have been reported in humans. In this study, three electrophoretic patterns were found in the dolphin

hemolysates. Forty-six of the 50 samples exhibited a major zone of activity just behind hemoglobin, and a second, weaker band of activity just behind this major zone. In three of the samples, the minor band was absent. One of the samples analyzed exhibited both the major and minor zones of activity, although the major zone had a slower electrophoretic mobility than in the other samples. These results are summarized in Figure 6.

G. Glyoxylase

Red cell glyoxylase I, also known as Lactoyl-Glutathione Lyase, catalyzes the irreversible conversion of gluthathione and methyl/glyoxal to S-lactoyl-glutathione. In man, three electrophoretic phenotypes of Glyoxylase 1 have been identified. These polymorphic phenotypes, referred to as GLO 1, GLO 2-1 and GLO 2, are determined by two, codominant allelic genes (GLO^1 and GLO^2). In humans, this GLO polymorphism is proving to be a promising genetic marker for population, genetic surveys and for paternity testing.

As illustrated in Figure 7, 90% of the dolphin hemolysates analyzed were of the GLO 2 phenotype, while 10% were found to be GLO 2-1. None of the samples examined were formed to be homozygous for GLO 1.

H. Haptoglobin

Haptoglobin is a glycoprotein found in serum whose physiological function is to combine with free hemoglobin. This rapid removal prevents deposition and damage to the renal tubes while preventing loss of iron via the kidney.